UNIVERSIDAD DE CÓRDOBA

FACULTAD DE VETERINARIA

ZOONOSIS ALIMENTARIAS EN EL CERDO IBÉRICO: PREVALENCIA Y CONTROL DESDE LA GRANJA PARA LA OBTENCIÓN DE ALIMENTOS SEGUROS

FOOD-BORNE ZOONOSIS IN THE IBERIAN PIG: PREVALENCE AND CONTROL FROM FARM FOR OBTAINING SAFE FOOD

Tesis presentada por la Licenciada en Veterinaria y en Ciencia y Tecnología de los Alimentos Dña. Ángela Morales Partera

para optar al Grado de Doctor en Veterinaria por la Universidad de Córdoba

Departamento de Sanidad Animal

Córdoba, 2019

TITULO: *Zoonosis alimentarias en el cerdo ibérico: prevalencia y control desde la granja para la obtención de alimentos seguros*

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Tesis Doctoral

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Esta tesis doctoral se ha realizado gracias al proyecto *SAFEPORK Zoonosis Alimentarias en el cerdo Ibérico: prevalencia y control para la obtención de alimentos seguros*, financiado con el Fondo Europeo de Desarrollo Regional, y gestionado por la Agencia de Innovación y Desarrollo de Andalucía (IDEA). En este proyecto intervienen el Centro Tecnológico de Investigación y Calidad Agroalimentaria del Valle de los Pedroches (CICAP), representado por el grupo PAIDI AGR-263 *Investigación Agroalimentaria*, y la Universidad de Córdoba, representada por dos grupos PAIDI de investigación el grupo AGR-137 *Anatomía Patológica Animal* y el grupo AGR-256 *Sanidad Animal: Diagnóstico y control de enfermedades*.

TÍTULO DE LA TESIS: ZOONOSIS ALIMENTARIAS EN EL CERDO IBÉRICO: PREVALENCIA Y CONTROL EN LA GRANJA PARA LA OBTENCIÓN DE ALIMENTOS SEGUROS

DOCTORANDO/A: Ángela Morales Partera

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

Esta tesis doctoral se presenta como compendio de publicaciones de los trabajos derivados del proyecto "*SAFEPORK Zoonosis Alimentarias en el cerdo Ibérico: prevalencia y control para la obtención de alimentos seguros*", financiado con el Fondo Europeo de Desarrollo Regional, y gestionado por la Agencia de Innovación y Desarrollo de Andalucía (IDEA) (referencia #351504).

El primer objetivo de esta tesis doctoral ha sido determinar la prevalencia de los distintos patógenos zoonósicos que afectan al cerdo Ibérico en la dehesa mediante estudio de los animales sacrificados en matadero. Para ello se ha desarrollado el trabajo (Morales-Partera et al., 2018, *Food Control*, 92, 208-215) que evaluó la prevalencia y diversidad de *Salmonella* spp., *Campylobacter* spp. y *Listeria monocytogenes* a lo largo de la cadena de producción del cerdo Ibérico en matadero

En base a los hallazgos encontrados en la presente investigación se demuestra la elevada prevalencia de *Salmonella* spp., *Campylobacter* spp. y *L. monocytogenes* en cerdos ibéricos criados en la dehesa, con la prevalencia más alta en amígdalas para *Salmonella* spp. (30,67%, IC95 23,85-38,44%) y *L. monocytogenes* (39,33%, CI95 31,87-47,32%) y en heces para *Campylobacter* spp. (57.33%, CI95 49.33-64.96%). Se observó una alta diversidad solo en los aislamientos de *Salmonella* de muestras de cerdos en el matadero, con evidencia de contaminación cruzada a lo largo de la cadena de producción en el caso de este patógeno. Las etapas previas al sacrificio (en la granja, transporte o la estabulación) y los procedimientos durante el sacrificio y faenado, incluyendo el sellado del recto antes de la evisceración intestinal y los protocolos de desinfección hidroalcohólica, se identificaron como estrategias de control críticas para reducir la contaminación de la carne de cerdo por *Salmonella* spp., *Campylobacter* spp. y *L. monocytogenes.*

El segundo objetivo ha consistido en aplicar medidas correctoras que permitan disminuir la prevalencia de los distintos agentes zoonósicos alimentarios de interés. Para esto se ha llevado a cabo el trabajo (Morales-Partera et al., 2019, actualmente en revisión). Este estudio evaluó el efecto de *Pediococcus acidilactici* como alternativa a los antibióticos y su impacto en los parámetros productivos y la microbiota fecal de cerdos en fase de engorde De este estudio deriva la conclusión de que la alimentación con una dieta suplementada con un aditivo a base de *Pediococcus acidilactici* MA18 / 5M en la fase de finalización logró reducir la carga de *Campylobacter* spp.en las heces de cerdos ibéricos criados en extensivo.

El tercer y último objetivo de esta tesis doctoral ha sido determinar la viabilidad de los patógenos seleccionados frente a las condiciones de curación empleadas en los productos del cerdo Ibérico. Para ello se llevó a cabo el trabajo (Morales-Partera et al., 2017, *Int J Food Microbiol*, 258, 68-72), en el que se analiza la eficacia del proceso de curación de los lomos de cerdos Ibéricos criados en extensivo en la reducción de tres de los patógenos más importantes transmitidos por los alimentos. Este estudio determinó que la concentración inicial de bacterias; la reducción progresiva del pH y la reducción de los valores de aw se consideran tres factores críticos en los cambios observados en *S. Typhimurium, C. coli* y *L. monocytogenes* a lo largo del proceso de curación de la caña de lomo de cerdo ibérico. Aunque se observa una reducción de los recuentos bacterianos durante todo el proceso, los resultados de este estudio no respaldan que el proceso de producción de lomos de cerdo curados en seco elimine completamente *S. Typhimurium y L. monocytogenes* en caso de alta contaminación inicial.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 30 de Septiembre de 2019

Firma de los directores

Fdo.: Carmen Tarradas Iglesias

Fdo.: Jaime Gómez Laguna

Fernando Cardoso

Fdo.: Fernando Cardoso Toset

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Capítulo I / Chapter I:

Introducción / Introduction

EL PAPEL DEL SECTOR PORCINO EN LA ECONOMÍA ESPAÑOLA

El sector porcino constituye un importante motor de la economía española, que representa en torno al 38 % de la Producción Final Ganadera y el 12,7 % de la Producción Final Agraria, con un volumen de facturación que roza los 6.000 millones de euros[1]. De este modo, el porcino supone uno de los sectores más importante de nuestra ganadería. Dentro de España, Andalucía ocupa el cuarto puesto en producción de carne de cerdo (7,6 %)[1].

Con una producción que supone el 16,9 % de la producida en la UE, España es considerada la cuarta potencia productora a nivel mundial (después de China, EEUU, y Alemania) y la segunda en Europa. Debido a que el sector porcino en Europa presenta un alto nivel de autoabastecimiento (alrededor del 111%), la exportación supone un elemento clave para el equilibrio del mercado, siendo la UE la principal potencia exportadora a nivel internacional. En este escenario, España se sitúa como tercer exportador de porcino de la UE, por detrás de Alemania y Dinamarca[1].

Según la encuesta de sacrificio del MAPAMA del año 2017 la carne de cerdo en nuestro país alcanza cifras record con más de 49,6 millones de animales sacrificados y unos 4,25 millones de toneladas de carne producida, experimentando en los últimos 5 años un elevado crecimiento como consecuencia del espectacular desarrollo del sector, claramente por encima de la media de la UE (Figura 1). La industria cárnica es el cuarto sector industrial de España, constituido por cerca de 3.000 empresas principalmente pequeñas y medianas (mataderos, salas de despiece e industria de elaborados) distribuidas por toda la geografía española, especialmente en zonas rurales. La producción de estas empresas hace que la industria cárnica ocupe con diferencia el primer lugar de toda la industria de alimentos y bebidas en España, representando una cifra de negocio de 26.207 millones de euros, el 22,1 % de todo el sector alimentario español (ANICE, 2019).

[1] http://www.magrama.gob.es/es/ganaderia/temas/produccion-y-mercados-ganaderos/sectores-ganaderos/porcino/

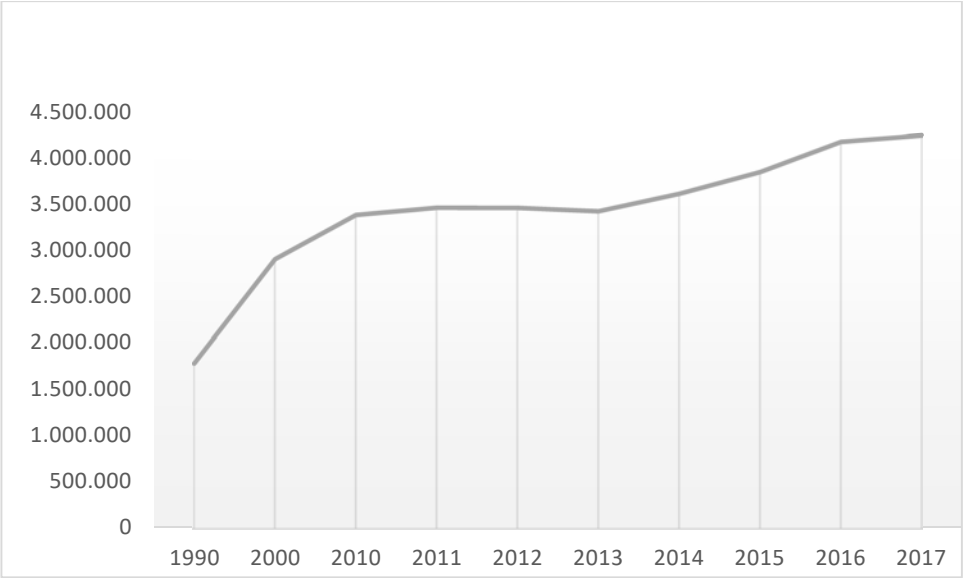


Figura 1. Producción española de carne de porcino según datos del MAPAMA (Ministerio de Agricultura, Pesca Alimentación y Medio Ambiente).

En este contexto, el sector porcino español se ha establecido como un amplio tejido industrial, formado por miles de pequeñas y medianas empresas repartidas por todo el territorio nacional, que proporcionan empleo a millones de familias (unos 175.000 empleos directos y unos dos millones de puestos de trabajo indirectos), actuando como un claro impulsor de la economía española (Buxadé, 2014). Al igual que las restantes producciones ganaderas, el sector porcino debe hacer frente a las nuevas exigencias del mercado, relacionadas con la seguridad alimentaria, el cuidado del medio ambiente y el bienestar animal (Langreo y González, 2007).

APROXIMACIÓN A LOS SISTEMAS DE PRODUCCIÓN PORCINA EXTENSIVA

Dentro del sector porcino existen dos subsectores claramente diferenciados y bien representados en nuestro país, los sistemas de producción *intensiva*, caracterizados por utilizar cerdos de razas mejoradas genéticamente (Landrace, Large White, Duroc, Pietrain, etc), criados en el interior de naves con un manejo nutricional basado en la utilización de alimentos concentrados o piensos compuestos, y los sistemas de producción *extensiva o semiextensiva*, en los que una parte importante del ciclo de cría y engorde de los animales ocurre en el exterior, aprovechando los recursos naturales (Paramio et al., 2012).

Algunos ejemplos de cría porcina extensiva fuera de nuestras fronteras son la cría del cerdo Alentejano en Portugal (Oliveira et al., 2014), el cerdo Negro de Nebrodi en Sicilia (Di Marco et al., 2012) o la cría de razas criollas en Latinoamérica (Velasco, 2006).

En España, el sistema de cría extensiva supone alrededor del 10% de la producción nacional y se encuentra estrechamente ligado al cerdo Ibérico y sus cruces, que comprende una amplia gama de variedades, líneas y estirpes genéticas (Paramio et al., 2012). En Andalucía el número de explotaciones en sistema extensivos (6.699) supera al de intensivo (5.595) a diferencia de otras Comunidades, por ejemplo en Galicia, donde sólo existen 70 ganaderías extensivas frente a 27.997 ganaderías intensivas (REGA 01/03/2018)[2].

Una particularidad del sistema extensivo en España es su vinculación al ecosistema de la dehesa (Figura 2), basada en un largo proceso de crianza y en el aprovechamiento de los recursos naturales durante la montanera, donde los cerdos se alimentan principalmente de bellota, pero también de pasto, raíces, insectos y pequeños vertebrados, dado el carácter omnívoro de la especie porcina (Astorga et al., 2010).

Si bien estos sistemas permiten a los animales gozar de un alto nivel de bienestar animal y la obtención de productos derivados de elevada calidad organoléptica (Ventanas, 2001), también favorecen el contacto entre distintas especies animales (Figura 3), tanto domésticas (ganado vacuno, caprino y ovino) como silvestres (ciervos, jabalíes, tejones, etc.) y condicionan que los animales se vean expuestos a múltiples factores ambientales que favorecen la diseminación de patógenos, sobre los cuales resulta difícil actuar (Hermoso de Mendoza et al., 2003; Astorga et al., 2010; Gortázar et al., 2011) y que pueden tener unas consecuencias graves, para el sector, de hecho en estudios previos demuestran la amplia dispersión de enfermedades de gran impacto sobre la producción porcina. (Galán-Relaño et al., 2015).

[2] https://www.mapa.gob.es/es/ganaderia/estadisticas/indicadoreseconomicossectorporcinoano2018_tcm30-379728.pdf

Figura 2. Cerdos Ibéricos criados en el ecosistema extensivo de la dehesa.

Una de principales características del ciclo epidemiológico y patogénico de los microorganismos estudiados en esta tesis doctoral es que los animales infectados no suelen presentan manifestaciones clínicas, pasando su estatus desapercibido para los veterinarios técnicos de la granja, si bien pueden ser portadores de dichos patógenos a distintos niveles, reactivándose su estado de latencia ante situaciones de estrés, principalmente en el transporte y sacrificio. En este sentido, cabe destacar que los mataderos presentan distintos puntos críticos de contaminación a lo largo de la cadena de sacrificio, desde donde estas bacterias acantonadas pueden alcanzar el producto final a través de contaminaciones cruzadas procedentes del contenido intestinal, piel, utensilios, agua e incluso a partir de manipuladores (Pearce et al., 2004; Buncic et al., 2012; Choi et al., 2013).

Es por tanto necesario establecer programas sanitarios y medidas de manejo para el control de las principales dominantes patológicas de este tipo de ganado, ocupando las enfermedades transmisibles, infecciosas y parasitarias, un lugar importante, no sólo por las pérdidas económicas que originan y su capacidad de diseminación dentro y entre granjas, sino también por las

repercusiones sobre la Salud Pública y la Seguridad Alimentaria, hechos que obligan a establecer sistemas de vigilancia y control a nivel nacional e internacional (Directiva 2003/99/CE de 17 de noviembre de 2003, sobre la vigilancia de las zoonosis y los agentes zoonósicos).

Figura 3. Coexistencia de distintas especies en el ecosistema de la dehesa.

ZOONOSIS ALIMENTARIAS Y LA CARNE DE CERDO

Las enfermedades zoonósicas transmitidas por los alimentos han sido tradicionalmente una amenaza para la salud pública mundial (Thorms, 2000), ante esta problemática, y como una de sus prioridades, la UE planteó la necesidad de garantizar un alto grado de seguridad alimentaria. Para ello, en diciembre del año 2000 publicó el Libro Blanco sobre seguridad alimentaria, que fue la base de mejoras legislativas organizativas y de coordinación entre los Estados Miembros. La Directiva *2003/99/CE de 17 de noviembre de 2003 sobre la vigilancia de las zoonosis y los agentes zoonósicos y el Reglamento (CE) 2160/2003 también de 17 de noviembre de 2003 sobre el control de la salmonela y otros agentes zoonósicos transmitidos por alimentos* recogen un enfoque integrado de la seguridad alimentaria, con un concepto que engloba toda la cadena de producción, *desde la granja hasta la mesa* (EFSA, 2018);

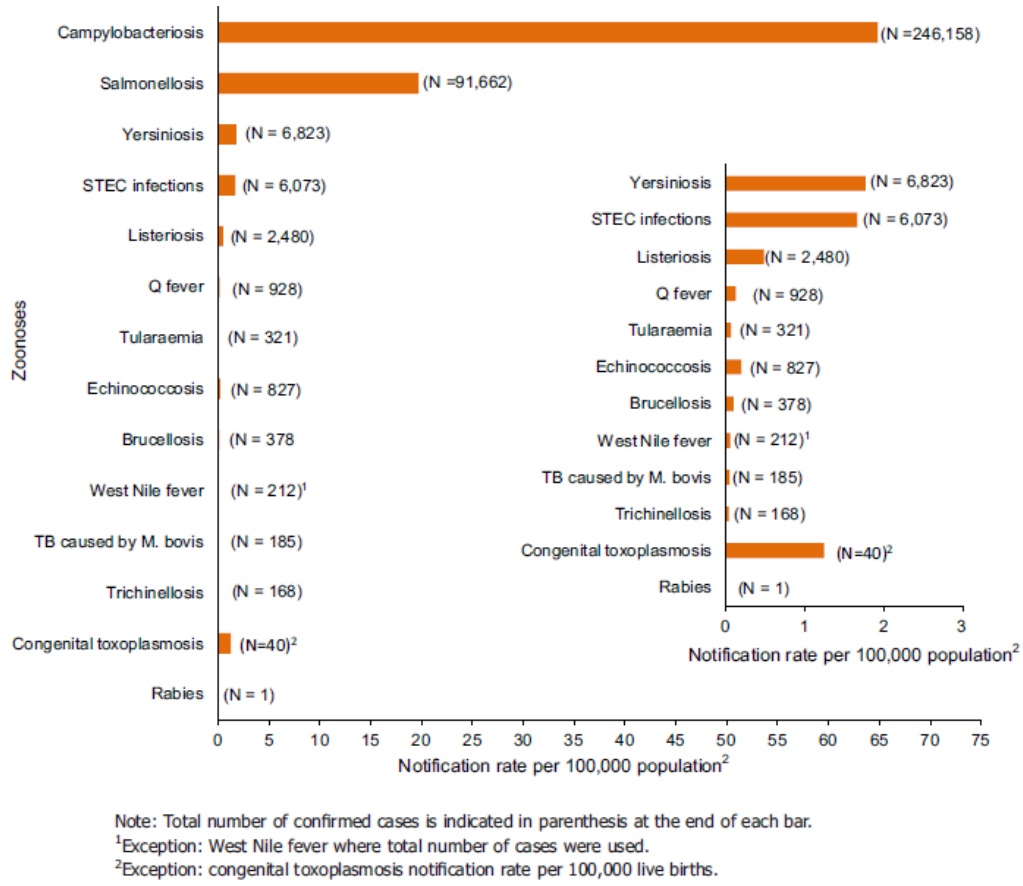
planteándose entre otros objetivos la identificación de las etapas de la producción de carne de cerdo y sus productos derivados que suponen un mayor riesgo para la salud pública.

A pesar de todo el esfuerzo llevado a cabo por las administraciones públicas y las empresas agroalimentarias en los países industrializados, se estima que hasta el 10 % de la población humana está en riesgo de sufrir alguna zoonosis alimentaria (Schlundt et al., 2004). Por ejemplo, en la UE, se han descrito un total de 5.079 brotes de origen alimentario durante el año 2017, que causaron 43.400 casos clínicos, 4.541 hospitalizaciones y 33 muertes (EFSA, 2018), aunque se estima que el número de afectados puede ser mucho mayor, ya que hay casos en los que no se realiza un diagnóstico final o no existe una vinculación directa con el alimento. Además de suponer un peligro para la salud, los brotes de enfermedades originados por alimentos generan grandes pérdidas económicas, debido, entre otras causas a las repercusiones comerciales que sufre la industria de los alimentos involucrados (Todd, 1989). Según los datos recogidos en el boletín de la EFSA de noviembre de 2018, entre los agentes más frecuentemente relacionados con brotes de enfermedad transmitidos a través de los alimentos se encuentran *Campylobacter* spp., *Salmonella* spp., *Yersinia* e*nterocolitica*, *Escherichia coli verotoxigénico* y *Listeria monocytogenes* (EFSA, 2018).

Existe un amplio número de alimentos que pueden ser transmisores de enfermedad para el hombre y, aunque la carne de cerdo está relacionada con un menor número de brotes de enfermedad que otros productos cárnicos, juega un papel importante debido a su elevado consumo y ser según las perspectivas agrícolas de la OCDE/FAO para 2027 la carne más consumida en la Unión Europea. Además, estudios llevados a cabo en varios países de la UE, demuestran que la carne de cerdo y los embutidos pueden actuar como fuente de infección de algunos microorganismos (Gómez et al., 2014; Holley y Cordeiro, 2014), destacando según algunos autores la importancia de *Listeria monocytogenes* (Modzelewska-Kapitula, 2014).

SITUACIÓN ACTUAL DE *Campylobacter* spp., *Salmonella* spp. Y *Listeria monocytogenes* COMO PATÓGENOS INDICADORES EN LA CADENA ALIMENTARIA

Tradicionalmente se ha considerado un grupo de agentes zoonósicos como los protagonistas indiscutibles de las Enfermedades Transmitidas por Alimentos (ETAs) entre los que se encuentran *Campylobacter* spp., *Salmonella* spp. y *Listeria monocytogenes*, entre otros (Figura 4).



Note: Total number of confirmed cases is indicated in parenthesis at the end of each bar.
[1]Exception: West Nile fever where total number of cases were used.
[2]Exception: congenital toxoplasmosis notification rate per 100,000 live births.

Figura 4. Números de casos reportados y tasas de notificación de zoonosis humanas confirmadas en la UE durante el año 2017 (EFSA,2018).

Campylobacter spp.

Campylobacter spp., es responsable de la mayoría de los brotes de origen alimentario con cuadros de gastroenteritis (EFSA, 2018). *Campylobacter* es una bacteria Gram negativa, perteneciente a la familia *Campylobacteraceae*, de la que se reconocen varias especies patógenas, siendo las especies *C. coli* y *C. jejuni* las más importantes para el hombre. Este microorganismo crece en un

rango de temperaturas muy amplio, entre 25 ºC y 42 ºC, siendo la temperatura óptima de 40-42 ºC, requiere pH neutro y valores de actividad agua (a_w) superiores a 0,998, con un metabolismo respiratorio microaerófilo pudiendo vivir con niveles bajos de oxígeno (Garrity et al., 2004).

Campylobacter jejuni se aísla con mayor frecuencia en carne de aves, aunque también se han encontrado niveles bajos en carne de cerdo (Schuppers et al., 2005; Fosse et al., 2009; Farzan et al., 2010), esta última responsable de la mayoría de los brotes causados por *Campylobacter coli* (Baer et al., 2013).

La campilobacteriosis es la enfermedad de origen alimentario más diagnosticada en humanos en la UE, por delante de las salmonelosis, con 246.158 casos confirmados (EFSA, 2018). En el 54,1 % de los casos confirmados en la UE, la información sobre las especies de *Campylobacter* aisladas muestra que la mayor parte son debidos a *Campylobacter jejuni* (84,4 %), el 9,2 % a *Campylobacter coli*, y el 0,1 % a *Campylobacter lari*, *Campylobacter fetus* y *Campylobacter upsaliensis* respectivamente; las otras especies de *Campylobacter* supusieron el 6,2 % de los casos confirmados (EFSA, 2018).

En productos alimentarios, *Campylobacter* se aísla con frecuencia a partir de carne de ave, fundamentalmente de carne fresca (37,4 %), aunque también se ha aislado a partir de carne de pavo, cerdo y ternera (31,5 %; 6,9 % y 1,4 %, respectivamente) (EFSA, 2018).

En este sentido, cabe señalar que desde enero de 2018 de acuerdo con el Reglamento 2017/1495 que modifica el Reglamento 2073/2005 se presta una mayor atención para este patógeno habiéndose establecido un límite de 1.000 ufc/g en las canales de pollo de engorde, su principal transmisor, como medida para reducir el número de casos de campilobacteriosis humana, y que podría llegar a hacerse extensivo a otros productos en un futuro, datos que aconsejan hacer un seguimiento en otras especies, como el cerdo.

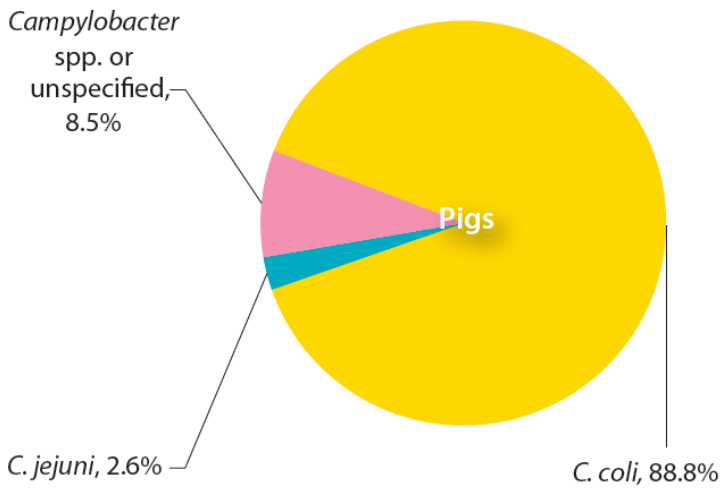


Figura 5. Distribución de especies de *Campylobacter* aislados de muestras positivas de cerdos en la UE en 2008.

En el cerdo, los estudios realizados en Europa demuestran la amplia dispersión de este patógeno en las ganaderías, detectándose mediante la aplicación de técnicas serológicas, cerca del 100 % de granjas positivas, con una seroprevalencia media de infección dentro de las granjas del 81,2 % (Fosse et al., 2009). Estudios previos realizados en el norte de España, aplicando técnicas de aislamiento han permitido determinar que la dispersión de la infección entre granjas es del 52,9 % y a nivel de individuos 57,8 % siendo *C. coli* la especie más frecuentemente aislada en el ganado porcino (Oporto et al., 2007), al igual que ocurre en la distribución de especies de *Campylobacter* en muestras de cerdos según datos de la EFSA (EFSA, 2008) (Figura 5).

Otros estudios demuestran una mayor prevalencia de *Campylobacter* a nivel de granja que a partir de carne fresca de cerdo (6,9 %) o productos "*ready to eat*" (RTE) derivados del cerdo (1,1 %), lo que indica que *Campylobacter* no es capaz de sobrevivir en la carne de porcino en el matadero durante las operaciones de sacrificio y procesado (EFSA, 2018).

Salmonella spp.

Salmonella spp. es una bacteria Gram negativa, perteneciente a la familia *Enterobacteriaceae*, de la que se reconocen numerosos serovares, siendo *S.* Enteritidis y *S.*Typhimurium los dos más frecuentemente aislados dentro de la

especie *S*. *enterica* subespecie *enterica*. Se trata de un microorganismo muy ubicuo que puede crecer en un amplio intervalo de temperaturas (de 5 a 45 ºC) y pH (de 4 a 9) y con valores de a_w por encima de 0,930 (Podolak et al., 2010).

Actualmente, la salmonelosis es la segunda enfermedad zoonósica más importante en la UE, después de la campilobacteriosis, con 91.662 casos clínicos confirmados en 2017. Aunque se ha observado una disminución significativa de los brotes de salmonelosis causados por *S.* Enteritidis en Europa desde 2008, parece que esta tendencia se ha estancado en los últimos 5 años (EFSA, 2018). Los brotes de salmonelosis se asocian en los últimos años, por orden decreciente, a los serotipos *S*. Enteritidis, *S*. Typhimurium, la variedad monofásica de *S*. Typhimurium (mST), *S*. Infantis y *S*. Newport (EFSA, 2018). Atendiendo al origen de las cepas los principales serovares de origen porcino detectados en casos humanos confirmados son *S*. Typhimurium, la variante mST. 4, [5], 12: i: - y *S*. Derby (Bonardi, 2017). En productos alimentarios, *Salmonella* se detecta con mayor frecuencia en huevos y derivados seguidos a continuación de la carne fresca de ave, pavo, cerdo y vacuno, con valores del 4,85 %, 4,18 %, 1,58 % y 0,17 % respectivamente, destacando entre éstas la carne picada y los preparados cárnicos (EFSA, 2018). Respecto a la carne de cerdo cabe señalar que se ha observado una fuerte asociación entre mST y la cadena de producción y sacrificio del ganado porcino (EFSA, 2018).

Estudios recientes muestran una alta prevalencia de *Salmonella* en la especie porcina, con valores de 36,1 %, 38,9 % y 44,7 % en instalaciones ganaderas porcinas, canales de cerdo en mataderos y corte de carne de cerdo en tiendas, respectivamente (Dang-Xuan et al., 2019). No obstante, recientemente se detectaron prevalencias más bajas en canales de mataderos italianos 12,3 % y 11,2 % (Bonardi et al., 2018).

La contaminación de las canales de cerdo puede ocurrir en la línea de sacrificio, y está relacionada con la contaminación cruzada de otras canales o la presencia de *Salmonella* en el medio ambiente. Por lo tanto, los serovares de *Salmonella* presentes en las canales de cerdos pueden ser diferentes de los detectados en la granja.

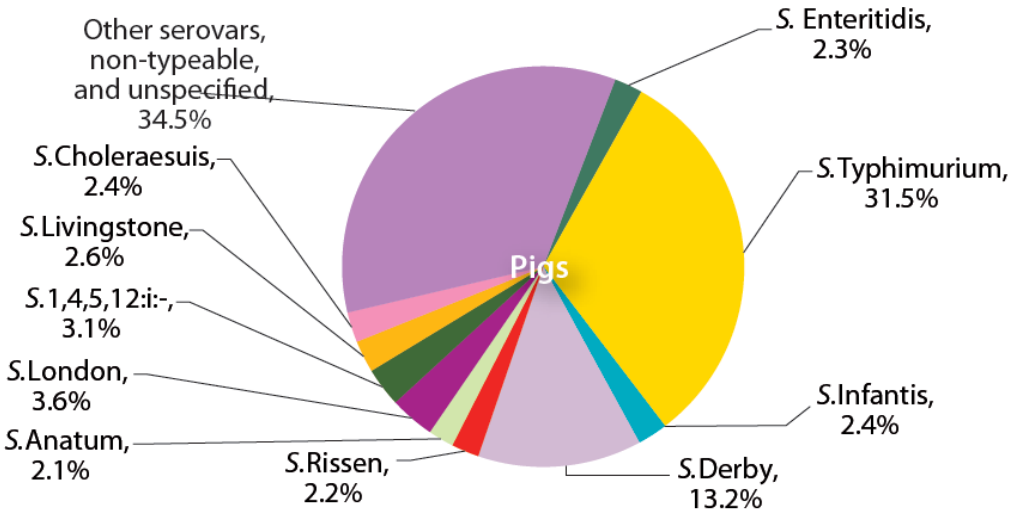


Figura 6. Distribución de los diez serotipos de *Salmonella* más comunes aislados en cerdos en la UE en 2008.

En granjas, la prevalencia de anticuerpos en Europa se sitúa en una 12,4 % de granjas infectadas y del 8,1 % de prevalencia de individuos infectados (Fosse et al., 2009). Mayor variabilidad muestran los estudios basados en el aislamiento de *Salmonella* spp., que por otra parte permiten determinar los serotipos implicados, ocupando *S*. Typhimurium la mayoría de los aislamientos a partir de cerdo blanco en Europa según datos de la EFSA 2008 (Figura 6). Estudios previos llevados a cabo en Andalucía han demostrado que la prevalencia de *Salmonella* es similar en sistemas de explotación intensivos y extensivos, aunque se han encontrado diferencias en cuanto a los serotipos implicados (Astorga et al., 2007; Gómez-Laguna et al., 2010), siendo en intensivo los más frecuentes *S*. Typhimurium, *S*. Derby y *S*. Rissen (Astorga et al., 2007) y en extensivo *S*. Typhimurium y *S*. Anatum (Gómez-Laguna et al., 2010).

Listeria monocytogenes

Otro agente de gran importancia para el hombre es *Listeria monocytogenes*, bacteria Gram positiva, perteneciente al género *Listeria* de la familia *Listeriaceae*, de la que se reconocen actualmente hasta 20 especies distintas (Leclercq et al., 2019). La mayoría de los casos de listeriosis en el hombre se deben al consumo de alimentos contaminados, y aunque existe una baja prevalencia, puede suponer una enfermedad grave en personas inmunodeprimidas causando cuadros nerviosos o septicémicos. Además, la infección en mujeres embarazadas puede dar lugar a abortos y malformaciones fetales (Baer et al., 2013). Esta bacteria es muy resistente a condiciones del medio adversas, crece en un rango de temperaturas muy amplio (-1 a 45 ºC), con un óptimo de 30 a 37 ºC, a pH entre 4 y 9,6 y en medios con a_w superiores a 0,90. Pueden crecer en alimentos con pH neutro y con un alto contenido de nutrientes a temperaturas alrededor de 0 ºC y puede sobrevivir a temperaturas de congelación de −18 ºC durante meses (Garrity et al., 2004).

Actualmente, la listeriosis es la quinta enfermedad zoonósica más importante en la UE, con 2.480 casos clínicos confirmados en 2017, un número muy inferior a los descritos para *Campylobacter* o *Salmonella*, destacando, sin embargo, por su gravedad clínica en personas no inmunocompetentes o inmunodeprimidas, llegando hasta un 13,8 % de mortalidad en el último año en la UE (EFSA, 2018). Aunque los animales domésticos pueden presentar signos clínicos como encefalitis, abortos, mastitis o septicemia, también pueden actuar como portadores intestinales asintomáticos y liberar el organismo en una cantidad importante, contaminando el medio ambiente o las canales durante el sacrificio y faenado en el matadero (EFSA, 2018). En España, los casos de listeriosis experimentaron un aumento progresivo desde 2013 a 2017, sin embargo se han visto reducidos en 2017 con respecto al 2016 (EFSA, 2018) (Figura 7).

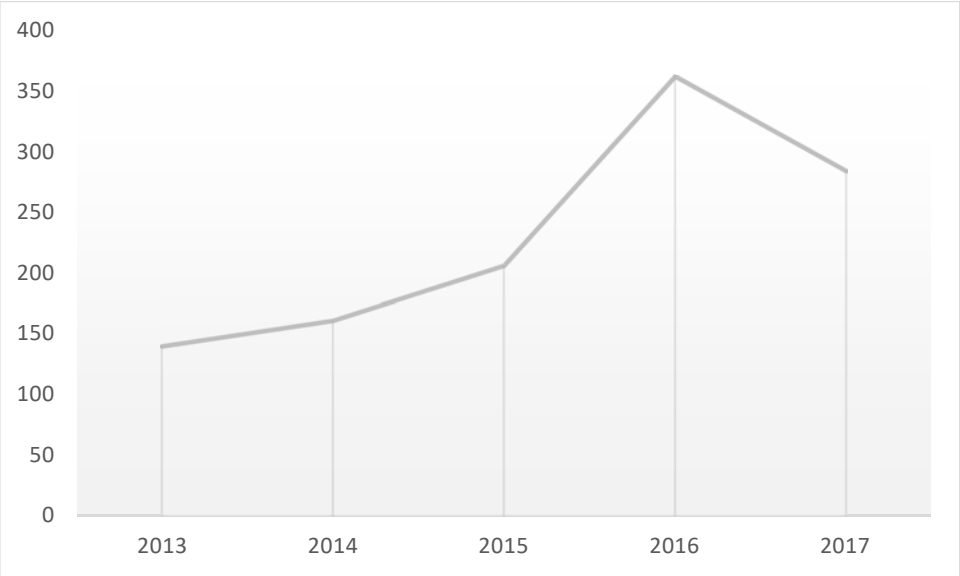


Figura 7. Evolución de casos de listeriosis en España desde 2013 a 2017 (EFSA 2018).

Entre las fuentes de infección de origen alimentario asociadas a este patógeno cabe destacar la importancia de los productos cárnicos listos para el consumo o "ready-to-eat" como los productos curados del cerdo. En este sentido, un estudio reciente llevado a cabo por la EFSA demostró la presencia de *L. monocytogenes* en 1,7 % de las muestras de productos cárnicos porcinos y 1,8 % de productos RTE de origen porcino (EFSA, 2018).

La información expuesta pone de manifiesto el interés y la importancia de desarrollar estudios encaminados a cuantificar la prevalencia de estos patógenos tanto a nivel de granja, como en matadero, sala de despiece y en producto final para poder determinar las medidas de control oportunas y conseguir así la obtención de productos cárnicos más seguros. Además, este control conllevaría una mejora del estatus sanitario de las explotaciones de cerdo Ibérico y una disminución de la prevalencia de agentes zoonósicos a nivel de matadero lo que supone un aumento de la rentabilidad y productividad del sector porcino.

ESTRATEGIAS DE CONTROL FRENTE A PATÓGENOS ZOONÓSICOS ALIMENTARIOS A NIVEL DE GRANJA

Existen múltiples evidencias de que los animales de granja pueden actuar como reservorio para muchos patógenos zoonósicos y que las explotaciones

ganaderas pueden ser una de las principales fuentes de infección para el hombre (Fosse et al., 2008; Lahuerta et al., 2011). Por lo tanto, la prevención de estas zoonosis transmitidas por los alimentos debe comenzar a nivel de granja (Cantas et al., 2014).

Entre las estrategias disponibles para ejercer este control se encuentra el empleo de aditivos alimentarios que ayuden a prevenir la infección de los animales con estos patógenos o a reducir su carga intestinal en animales portadores (Berry et al., 2016).

Hasta la actualidad se han utilizado varios aditivos de origen natural como los polifenoles, acidificantes, aceites esenciales y ácidos grasos omega-3. El uso de aditivos fitogénicos destaca por la estimulación del consumo y de la digestión y su poder antimicrobiano, antiinflamatorio, antioxidante, inmunomodulador, entre otros (Castro et al., 2005). Estudios con animales de laboratorio han mostrado una mejora en la respuesta inmune de los animales alimentados con polifenoles (Hocquette et al., 2009). El uso de polifenoles solubles en el pH ácido del estómago de los animales garantiza un efecto positivo a nivel intestinal, impide las presencia masiva de proteínas no digeridas a nivel del intestino grueso, uniéndose a ellas y evitando su uso por microorganismos patógenos, frenando su proliferación y la producción de diarreas y cuadros entero-tóxicos, lo que ayuda a la regulación de la flora intestinal y a paliar las diarreas post-destete de los lechones. En este sentido, los polifenoles también son conocidos por ejercer en condiciones *in vitro* funciones antimicrobianas frente a patógenos de interés, como *L. monocytogenes*, *S.* Typhimurium, *E. coli* O157:H7 o *Staphylococcus aureus*, entre otros (Hammer et al., 1999; Burt, 2004; Gulluce et al., 2007, Dorman y Deans, 2008). Los aceites esenciales o sus combinaciones han mostrado su carácter bacteriostático frente a *E. coli* en lechones (Laine et al., 2008) y estudios *in vitro* evidencian efecto frente a *Salmonella* entérica (Solarte et al., 2017). Sin embargo, una desventaja importante de los extractos de plantas se encuentra en el hecho de que sus composiciones son inestables debido a la influencia de muchos factores como el clima, la estación y el método de recolección entre otros (Borovan et al., 2004; Bomba et al., 2006). Probablemente por esta razón hay múltiples resultados controvertidos obtenidos en diferentes estudios científicos que examinan el efecto de estas sustancias en

la alimentación animal, siendo necesario seleccionar de manera eficaz extractos de plantas, estandarizarlos e investigar un beneficio potencial sinérgico derivado de su combinación (Budzinski et al, 2000; Lotufo et al, 2006). Por último, el uso de ácidos grasos ha demostrado reducir la respuesta inflamatoria (Korver y Klasing, 1997), favorece la producción de inmunoglobulinas específicas (Golian et al., 2010) e influye en la microbiota intestinal modulando la capacidad de colonizar el intestino (Kankaanpaa et al., 2001; Hekmatdoost et al., 2008).

Otra tendencia dentro de los aditivos alimentarios es el uso de bacterias ácido lácticas (BAL) dados sus efectos beneficiosos, también descritos en cerdos, entre los que se encuentran la regulación de la microflora intestinal, la inhibición de la proliferación de patógenos en el tracto gastrointestinal, el aumento de la inmunidad de la mucosa intestinal y el mantenimiento de la función de la barrera intestinal (Goktepe et al., 2005). Las BAL son un grupo de bacterias Gram-positivas tolerantes al ácido, que pertenecen a un género particular de bacterias, que se asocian por sus características metabólicas y fisiológicas y que producen ácido láctico como metabolito final de la fermentación de hidratos de carbono, así como otros ácidos orgánicos capaces de crear un microambiente ácido que inhiben el crecimiento de bacterias patógenas mediante la reducción de pH (Ridwan et al., 2008; Tejero-Sariñena et al. 2012). Asimismo, las BAL pueden inhibir activamente la proliferación de bacterias patógenas mediante la producción de bacteriocinas (O'Shea et al., 2012; Da Silva Sabo et al., 2014). Las bacteriocinas son péptidos bacterianos que ejercen su acción antibacteriana mediante diferentes mecanismos inhibitorios incluyendo la formación de poros en la membrana (Beasley et al., 2004), DNasa (Riley et al., 2006; Roh et al., 2010), la nucleasa (Chan et al., 2011) y la inhibición de la producción de mureína (Braun et al., 2012). A la hora de la selección de una BAL como probiótico, destacan dos parámetros en los que hay que fijarse: (i) la supervivencia en el tracto gastrointestinal superior, mostrando una alta resistencia a los ácidos inorgánicos (es decir, ácido clorhídrico), sales biliares y enzimas pancreáticas (Pringsulaka et al., 2015); y (ii) la capacidad de adherirse a las células epiteliales intestinales (Kadlec y Jakubec, 2014, Lähteinen et al., 2010), que favorezca la colonización bacteriana, la exclusión de patógenos y las interacciones de la célula huésped (Lebeer et al., 2008). En este sentido, *Pediococcus acidilactici*

ha sido señalado como candidato de interés como probiótico en cerdos (Lessard et al., 2009; Todorov et al., 2011; Balgir et al., 2013; Fernández et al., 2014; Barbosa et al., 2015; Kiran et al., 2015), ya que se ha demostrado que actúa compitiendo por los sitios de unión con otras bacterias patógenas (Ganan et al., 2012) y produce pediocinas con actividad frente a bacterias, como *Listeria*, *Clostridium*, *Bacillus*, *Staphylocoocus* o *Enterococcus* (Niamah, 2018).

VIABILIDAD DE *Campylobacter* spp., *Salmonella* spp. Y *Listeria monocytogenes* A LOS PROCESOS DE CURACIÓN Y TRATAMIENTOS ALTERNATIVOS

Los embutidos son productos elaborados mediante un proceso tecnológico que incluye adición de aditivos (fundamentalmente sal, sales nitrificantes y adobo) y una posterior maduración en cámaras sometidas a condiciones de temperatura y humedad controladas (Soto et al., 2008). Para garantizar la inocuidad de estos productos se pueden incorporar durante el procesado determinados aditivos con actividad antimicrobiana.

En este sentido, durante siglos se han utilizado sales, azúcares y ácidos se han utilizado no sólo para reducir la carga bacteriana sino también para mejorar el sabor del producto (Taormina et al., 2010; Hospital et al., 2012, Hospital et al., 2014; Tohora et al., 2014) y prolongar su vida útil.

Existen muchas investigaciones sobre tratamientos post-letalidad y agentes (sales y ácidos orgánicos) que inhiben el crecimiento de *L. monocyotogenes*. Así, el lactato sódico, diacetato sódico, ácido láctico y ácido acético tienen efectos antimicrobianos sobre *L. monocytogenes* usados solos o en combinación (Serdengecti et al., 2006; Georgnaras et al., 2006)

El ácido láctico y sus sales se han utilizado frecuentemente en la industria cárnica para potenciar su flavor y prolongar la vida media de los productos. Se ha descrito su actividad frente a *Clostridium botulinum*, *L. monocytogenes*, *Staphylococcus aureus*, *Salmonella* y *E. coli* O157:H7 (Smigic et al., 2010; Fouladkhad, 2013).

Otra estrategia es el empleo de bacteriófagos, virus específicos que atacan a las bacterias pero que son completamente inocuos para el hombre, los animales y el medioambiente. Estos constituyen un potencial para ser el siguiente gran avance tecnológico en agentes antibacterianos, pudiéndose utilizar como alternativa natural para garantizar que los productos finales no estén contaminados con patógenos como *Listeria* (Oliveira et al., Soni et al., 2014), *Salmonella* (Grant et al., 2017), *Campylobacter* (Grant et al., 2016) o *E. coli* (Hong et al., 2014).

En trabajos previos sobre productos curados derivados del cerdo se ha comprobado que la reducción de pH y a_w son los factores más importantes para conseguir un producto estable y seguro (Fadda et al., 2010), pero son escasos los trabajos donde se haya valorado la acción de los aditivos utilizados y el comportamiento de agentes patógenos concretos durante el proceso de curación para la obtención de productos RTE, como la caña de lomo Ibérica, uno de los productos con mayor demanda nacional e internacional de nuestros mercados (Cardoso-Toset et al., 2017; ANICE, 2019).

El efecto de las altas presiones sobre el procesado de los productos cárnicos representa otra alternativa a los tratamientos tradicionales térmicos y/o químicos para la conservación de alimentos de distinta naturaleza, incluyendo la carne de cerdo (Lavieri et al., 2014); este proceso tiene la característica de ser un proceso natural, respetuoso con el medioambiente y que permite preservar al máximo los ingredientes y características del producto fresco, pero puede presentar efectos no deseados en cuanto a las características sensoriales (Santos et al. 2013, Klug et al., 2017)

Un recuento de microorganismos fuera de los límites establecidos por el R.D 2073/2005 para la liberación de un producto alimentario al mercado origina una parada en su comercialización y en el caso de no ser detectado y liberado al mercado un riesgo para la salud pública. Las tecnologías usadas en la actualidad para determinar los recuentos de microorganismos suelen ser metodologías tradicionales de recuentos en placas con medio de cultivo que suponen un tiempo de espera de entre 72-120 horas. Aunque existan metodologías más rápidas, como es el caso de la PCR (Polymerase chain

reaction), ambas requieren el traslado de la muestra al laboratorio y son métodos destructivos lo que supone tiempo y dinero. Además son métodos destructivos que limitan el muestreo en un lote de producción dado su coste, si tenemos en cuenta el valor económico de algunos productos cárnicos como aquellos derivados del cerdo Ibérico. Por tanto el desarrollo de sensores que permitan la medición "*on line"* de la contaminación bacteriana de manera rápida, económica y no destructiva, y que por tanto permita incrementar la presión de muestreo, representa una de las grandes necesidades para las industrias cárnicas que permitirá asegurar la calidad de sus productos, incrementar la eficiencia de su producción dada la reducción de costes analíticos, disminución de tiempos de espera en la liberación del stock al mercado y por tanto aumento de la competitividad de la empresa.

En este sentido, estudiar el desarrollo de sensores dieléctricos y métodos de ensayos no destructivos aplicados al control y seguridad de los productos alimentarios de calidad es una disciplina científica cada vez con mayor interés en el sector agroalimentario (Blakey et al., 2016). Esto se debe a que la espectroscopia dieléctrica es un método sensible, asequible, de bajo consumo y no destructivo, lo que la hace ideal para el análisis continúo de los alimentos. Debido al rápido desarrollo y la disminución del tamaño de los teléfonos móviles los componentes de la tecnología microondas se han vuelto extremadamente pequeños y baratos en la última década. Esto hace que la metodología de los análisis dieléctricos que utilizan una frecuencia reducida sea mucho más asequible que tecnologías tales como la espectroscopia Near Infrared Spectroscopy (NIRs) espectroscopia Raman, fluorometría y espectroscopía de resonancia magnética nuclear (Blakey et al., 2016). Se espera que en el futuro este nuevo sistema de análisis reemplace los métodos de análisis más convencionales en el control de calidad de la industria agroalimentaria. Sus aplicaciones recientes incluyen la verificación de la calidad del aceite de oliva (Lizhi et al., 2010, Blakey et al., 2012), la cuantificación de bacterias (Blakey et al., 2013, Asami et al., 2014) y el análisis del contenido de agua de los alimentos (Martín-Esperanza et al., 2006, Sólyom et al., 2013).

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Capítulo 2 / **Chapter 2:**

Objetivos / Objectives

El **objetivo principal** de esta Tesis Doctoral consiste en determinar en el cerdo Ibérico criado en extensivo la prevalencia de los principales patógenos que intervienen en las zoonosis alimentarias y establecer medidas básicas de control en las distintas fases del sistema productivo (producción primaria y sacrificio) para reducir así su incidencia.

Objetivo específico 1: Determinar la prevalencia de los distintos patógenos zoonósicos que afectan al cerdo Ibérico en la dehesa mediante estudio de los animales sacrificados en matadero:

Estudio 1: "Prevalence and diversity of *Salmonella* spp., *Campylobacter* spp., and *Listeria monocytogenes* in two free-range pig slaughterhouses"

(Morales-Partera et al., 2018, *Food Control*, 92, 208-215)

Objetivo específico 2: Aplicar medidas correctoras que permitan disminuir la prevalencia de los distintos agentes zoonósicos detectados por su relación con la prevalencia de agentes zoonósicos alimentarios:

Estudio 2: "Effect of the probiotic *Pediococcus acidilactici* MA18/5M strain in reducing faecal *Campylobacter* spp. counts in free-range finishing pigs prior to slaughter"

(Morales-Partera et al., 2019, *en revisión*)

Objetivo 3: Determinar la viabilidad de patógenos seleccionados frente a las condiciones de curación empleadas en los productos del cerdo Ibérico:

Estudio 3: "Viability of selected foodborne pathogens in dry cured pork loins"

(Morales-Partera et al., 2017, *Int J Food Microbiol*, 258, 68-72)

The main objective of this Doctoral Thesis is to determine the prevalence of the main pathogens involved in foodborne zoonoses in the Iberian pig, and to establish basic control measures in the different phases of the production system (primary production and slaughterhouse) to reduce thus its incidence.

Specific objective 1: To determine the prevalence of the different zoonotic pathogens that affect the Iberian pig in the dehesa by studying slaughtered animals:

Study 1: "Prevalence and diversity of *Salmonella* spp., *Campylobacter* spp., and *Listeria monocytogenes* in two free-range pig slaughterhouses"

(Morales-Partera et al., 2018, *Food Control*, 92, 208-215).

Specific objective 2: Apply corrective measures to reduce the prevalence of zoonotic agents detected along the production chain:

Study 2: "Effect of the probiotic *Pediococcus acidilactici* MA18/5M strain in reducing faecal *Campylobacter* spp. counts in free-range finishing pigs prior to slaughter"

(Morales-Partera et al., 2019, *under review*)

Objective 3: To determine the viability of selected pathogens against the curing conditions used in Iberian pig products:

Study 3: "Viability of selected foodborne pathogens in dry cured pork loins"

(Morales-Partera et al., 2017, *Int J Food Microbiol*, 258, 68-72)

Capítulo III / Chapter III:

Estudios / Studies

Estudio 1 / Study 1

Prevalence and diversity of *Salmonella* spp., *Campylobacter* spp., and *Listeria monocytogenes* in two free-range pig slaughterhouses

Morales-Partera et al., 2018, Food Control, 92, 208-215

Objetivo específico 1: Determinar la prevalencia de los distintos patógenos zoonósicos que afectan al cerdo Ibérico en la dehesa mediante estudio de los animales sacrificados en matadero.

Prevalence and diversity of *Salmonella* spp., *Campylobacter* spp., and *Listeria monocytogenes* in two free-range pig slaughterhouses

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Abstract

Salmonella spp., *Campylobacter* spp. and *Listeria monocytogenes* have a significant impact on public health with slaughterhouses providing many opportunities for the proliferation of pathogenic bacteria. To evaluate the prevalence and diversity of these microorganisms along the free-range pork production chain, a total of 750 samples (5 samples/animal; 15 animals/farm; 5 farms/slaughterhouse) were collected from two slaughterhouses and analysed by specific ISO methodologies. *Salmonella* spp. (12.93%, CI_{95} 10.72-15.52%), *Campylobacter* spp. (17.17%, CI_{95} 13.00-21.74%) and *L. monocytogenes* (9.07%, CI_{95} 7.21-11.33%) were recovered at different stages of the production chain, with the highest prevalence detected in tonsils for *Salmonella* spp. (30.67%, CI_{95} 23.85-38.44%) and *L. monocytogenes* (39.33%, CI_{95} 31.87-47.32%) and in faeces for *Campylobacter* spp. (57.33%, CI_{95} 49.33-64.96%). Thirteen different *Salmonella* serotypes were detected with monophasic *Salmonella* Typhimurium as the most frequent one. *C. coli*, *C. jejuni* and *L. monocytogenes* serotype 4b and 1/2a were also identified. A significant higher prevalence of *Salmonella* spp. in total and from skin samples in slaughterhouse B than in slaughterhouse A was detected. In addition, a higher, although not significant, prevalence of the selected pathogens was observed in meat samples from slaughterhouse B with respect to slaughterhouse A (10.67% vs 0% for *Campylobacter* spp.; and 4% vs 0% for *Salmonella* spp. and *L. monocytogenes*). Our results highlight the risk of contamination of pork meat by the microorganisms under study and point out the importance of implementing specific control measures.

Keywords: *Salmonella*; *Campylobacter*; *Listeria monocytogenes*; free-range pig; pork production chain; slaughterhouse.

1. Introduction

Food-borne pathogens are of major health and economic significance in developed countries (Fosse et al., 2009; Fredriksson-Ahomaa et al., 2009). To reduce transmission to humans, it is important to identify which animals and foodstuffs are the main sources of the causative agents. *Campylobacter* spp. and *Salmonella* spp. are the most frequently reported zoonoses in 2016 in the European Union with notification rates of 66.3 and 20.4 cases per 100,000 population, respectively (EFSA, 2017). Listeriosis caused by *Listeria monocytogenes* increased in 2016 in comparison with 2015 (0.47 cases per 100,000 population). Despite presenting a lower incidence than the other pathogens, listeriosis is the most harmful one in the elderly, pregnant woman and immunocompromised individuals, with the highest hospitalization and mortality rate since 2008 (EFSA, 2017).

Pigs can be asymptomatic carriers of *Salmonella enterica*, *Campylobacter* and *L. monocytogenes*, and these pathogens can be isolated from the intestinal tract and tonsils of pigs (Fredriksson-Ahomaa et al., 2009; Farzan et al., 2010). These animals may be a source of contamination for other pigs and pork meat through the pork production chain (Hellström et al., 2010; Argüello et al., 2013; Prencipe et al., 2012; Hernández et al., 2013).

Different risk factors have been associated with the presence of these pathogens at farm level (Hellström et al., 2010), just like the slaughtering process has been related to the spreading and final contamination of pork derived products (Argüello et al., 2013; Hernández et al., 2013). In this sense, the molecular tracking of isolates by different techniques, such as Pulsed Field Gel Electrophoresis (PFGE), along the pig production chain allows monitoring the potential cross contamination of meat by several food-borne pathogens (Prenciple et al., 2012; Argüello et al., 2013; Hernández et al., 2013).

To reduce the occurrence of this type of zoonosis, it is important to identify which stages of the pork production chain pose a major risk for bacterial transmission. Slaughterhouses in particular provide many opportunities for pathogenic bacteria proliferation with contamination arising from the air, animal hide, utensils, water and slaughter equipment during bleeding, polishing, splitting, scalding and forced chilling (Pearce et al., 2004, Pearce et al., 2006; Bunic and Sofos, 2012; Choi et al., 2013). In this sense, a large number of animals for slaughter together with insufficient and/or irregular sanitation measures as well as post-harvest processing may be significant sources of bacterial introduction leading to carcass cross contamination (Delhalle et al., 2009; Papadopoulou et al., 2012; Hernández et al., 2013).

Nowadays there is growing interest for organic and eco-friendly pig rearing systems, but also for food quality and safety. Whereas free-range systems may allow a higher animal welfare status, food safety may be threatened by more limited biosecurity measures (Funk and Gebreyes, 2004). In a previous study we verified the risk of *Salmonella* spp. infection or recontamination prior and post slaughter and proposed control measures to reduce the final contamination of meat (Hernández et al., 2013). In this work the prevalence and diversity of *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes* in two free-range pig slaughterhouses is analysed as well as the effectiveness of the control measures implemented in each slaughterhouse.

2. Materials and Methods

2.1 Sample collection

Ten farms located in two different regions of southwestern Spain (Andalusia) were selected for this study based on their high *Salmonella* spp. seroprevalence (from 80.0% to 100.0% of individual seroprevalence; cut-off 40 OD%; SALMOTYPE® Pig Screen, Labor Diagnostik Leipzig, Leipzig,

Germany). The distance among farms within each region was always lower than 30 km. Each farm had an average of 200 hundred finisher pigs. All evaluated pigs (15 pigs/farm; 150 animals) were raised in free-range conditions, reared outdoors in sparse oak forests (dehesa) where they fed on acorns and grass and share natural resources with other wild and domestic animals. Sample collection was performed in two slaughterhouses (slaughterhouse A and slaughterhouse B, with five farms per slaughterhouse) from January to March 2013.

Only free-range pigs were slaughtered in both abattoirs, with an average of 1,500 pigs/slaughtering, coinciding always with the timeframe from September to June next year. During this working period a systematic cleaning and disinfection protocol is carried out following each slaughtering. From June to September, wherein no slaughter was performed, more exhaustive and meticulous cleaning and disinfection protocols of the abattoir are carried out, including the dismantling of the equipments. In addition, more thorough cleaning strategies and sanitation processes including the routine sealing of the rectum before gut evisceration and intensification of hydroalcoholic disinfection protocol applied on the cutting surface at quartering in spray at approximate intervals of 4 hours were performed in slaughterhouse A. Both slaughterhouses used gaseous asphyxiation to stun animals before bleeding, scalding of animals in a vertical scalding tunnel with a showered system with hot water at 60 ± 1 ºC, dehairing with a dehairing machine and singeing with a blow lap. Pigs were slaughtered following Good Manufacturing Practices (GMP), Sanitation Standard Operating Procedures (SSOP) and Hazard Analysis Critical Control Point (HACCP) under veterinary supervision and the traceability throughout the slaughter was strictly followed. Five samples per animal were aseptically collected at different stages of the production chain and transported into sterile containers to laboratory as follows: i) post-stunning/pre-scalding skin sample using an abrasive sponge; ii) post-evisceration, ileocecal lymph nodes and iii) faeces; iv) tonsils samples; and v) quartering, a pool of meat samples from ham, loin and shoulder. According to previous studies, the results of tonsils, lymph nodes, skin and faeces were

considered to be indicative of the pig infection status before the slaughter process (on the farm, during transport or in lairage) and meat samples results were considered to be informative about hygiene during the slaughter process (Swanenburg et al., 2001b). The choice of the ileocecal lymph nodes was carried out to allow detecting pigs in a carrier state (Argüello et al., 2013). Sterile dehydrated sponges were pre-moistened with 10 ml of sterile peptone water and the target surface (skin from shoulder, back and ham) was swabbed by using an overlapping S pattern to cover the entire surface. The surface of the complete tonsils and ileocecal lymph nodes were decontaminated using 100 % ethanol and flaming to eliminate extrinsic bacteria. Then, to avoid the possible effect of non-homogeneous distribution of the evaluated bacteria throughout these samples, complete tonsils and ileocecal lymph nodes were individually cut into small pieces and mixed using sterile scalpels before analysis.

For each bacterial analysis one pre-moistened abrasive sponge, 1 g of tonsil homogenate, 1 g of ileocecal lymph nodes homogenate, 25 g of faeces and 25 g of meat homogenate were independently processed. In order to avoid an excessive slowing-down of the slaughter line, abrasive sponges for *Campylobacter* analysis were not collected.

2.2 Bacterial isolation and identification

All the isolates were isolated and identified according to specific ISO methodologies. When confirmed, pure cultures of each isolate were stored at - 80 ºC on Microbank beads (Pro-Lab Diagnostics, Spain).

2.2.1. *Salmonella* isolation and typing

All samples were analysed by using specific ISO methodologies for the detection of *Salmonella* spp. (ISO 6579:2002). Sterile peptone water (Scharlab, Spain) was added to each sample at a 1:10 ratio and incubated at 37 ºC for 24 hours. Samples were homogenised in a Masticator Classic (IUL

S.A, Spain) for 1 minute at 1500 rpm before incubation. After incubation, pre-enriched culture was transferred to Rappaport-Vassiliadis Medium Semisolid Modified (MRSV) (Oxoid, UK) and incubated at 42 ºC for 48 hours. Isolates were cultured on Xylose lysine deoxycholate agar (XLD) and *Salmonella* Chromogenic agar base (Oxoid, UK) and incubated at 37 ºC for 24 hours. All presumptive *Salmonella* isolates were biochemically confirmed by lysine iron agar (Difco, Spain), Kligler's iron agar (Oxoid, UK) and motility indole ornithine agar (Difco, Spain).

Bacteria serotypes and phage types were determined by means of an agglutination technique using commercially available polyvalent and monovalent *Salmonella* antisera against O (somatic) and H (flagellar) antigens (BioRAD, Spain) and specific phage types to type *S*. Typhimurium and mST provided by the International Reference Laboratory of phage typing (Colindale, UK) at the National Microbiology Centre Institute of Health Carlos III (Madrid, Spain).

2.2.2. *Campylobacter* isolation and typing

All samples were analysed by using specific ISO methodologies for detection of *Campylobacter* spp. (ISO10272-1:2006). Bolton broth supplemented with lysed horse blood (Oxoid, UK) was added to each sample at a 1:10 ratio, homogenised with a Masticator Classic (IUL S.A, Spain) for 1 minute at 1500 rpm and incubated in microaerophilia using GENbox microaer atmospheric generators (bioMérieux, France) at 42 ºC for 48 hours. Then, a loopful of inoculum was spread onto selective medium CASA agar (bioMérieux, France) and incubated under the same conditions. Faeces were directly inoculated onto CASA agar (OIE, 2017). After visual inspection, presumptive *Campylobacter* colonies (a maximum of five representative colonies per sample) were subcultured onto non-selective Columbia agar (bioMérieux, France) and incubated under the same conditions. Morphology and motility, oxidase, catalase, detection of hippurate hydrolysis and indoxyl hydrolysis, and the latex agglutination technique (AES Chemunex, France)

were used for the preliminary identification of enteropathogenic, thermophilic *Campylobacter*, according to ISO 10272-1:2006. Finally, a species-specific multiplex PCR assay was performed to identify *Campylobacter* isolates as *Campylobacter coli, Campylobacter jejuni* or *Campylobacter* spp. (Yamazaki et al., 2008).

2.2.3. *Listeria monocytogenes* isolation and typing

Samples were analysed by using specific ISO methodologies for detection of *L. monocytogenes* (ISO11290-2:2000). Sterile peptone water (Scharlab, Spain) was added to each sample at 1:10 ratio, homogenised with a Masticator Classic (IUL S.A, Spain) for 1 minute at 1500 rpm and incubated at 37 ºC for 24 hours. After incubation, pre-enriched culture was transferred to ALOA agar (bioMérieux, France) and incubated at 37 ºC for 24 hours. Presumptive colonies identified as *L. monocytogenes* were serotyped using a commercial Listeria Antisera Set (O and H antigens; Denka Seiken Co., Tokyo, Japan) following to the manufacturer's instructions.

2.3 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was used to characterise the genotype of *Salmonella* spp. and *L. monocytogenes* when the same serotype was isolated from meat and other source samples within the same sampling. *Campylobacter* isolates were not successfully recovered after freezing storage and could not be subjected to PFGE analysis.

Genotyping of *Salmonella* was done after genomic DNA digestion with *Xba*I (Life technologies, USA) as previously reported (Hernández et al., 2013). *Listeria monocytogenes* isolates were subjected to PFGE using the restriction enzyme *Bsp*120I (Life technologies, USA) according to a previously published protocol (Graves and Swaminathan, 2001). PFGE technique was performed by using a CHEF-DRIII System (Bio-Rad Laboratories, USA).

PFGE patterns (PFP_s) were analysed by means of InfoQuest FP software version 4.5 (Bio-Rad Laboratories, USA). Clustering of patterns was evaluated by the unweighted-pair group method with arithmetic averaging and the Dice coefficient. The Dice similarity coefficient was used with optimization and position tolerance setting of 0.5 and 1.5 %. Only strains with an indistinguishable profile were considered to represent a single clone.

2.4. Statistical analysis

A descriptive study was carried out at the different sampled stages in each slaughterhouse (post-stunning/pre-scalding: superficial abrasive sponge samples; post-evisceration: ileocecal lymph nodes, faeces and tonsils samples; quartering: meat samples) and the 95 % confidence interval (CI_{95}) was assessed by Wilson method using the software Winepi (http://www.winepi.net/) (Veterinary Medicine Faculty, University of Zaragoza, Spain). Then, a transversal observational analysis was performed comparing the different pathogens as well as the different stages of the production chain examined in each slaughterhouse to determine the role of the slaughterhouse and each stage as a protection or as a risk factor (χ^2 estimate, prevalence ratio, and χ^2 approximation; Winepi); results with a *P* value ≤ 0.05 were considered as statistically significant.

3. **Results**

3.1. Salmonella spp., Campylobacter spp. and L. monocytogenes prevalence

Table 1 summarizes the number of positive samples and the prevalence at each sampling point for *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes* in each slaughterhouse and in total. A total of 750 different samples were tested for *Salmonella* spp. and *L. monocytogenes*. Six hundred samples were collected and tested for *Campylobacter* spp. *Salmonella* spp. was isolated from 97/750 samples (12.93 %, CI_{95} 10.72-15.52 %),

Campylobacter spp. from 103/600 samples (17.17 %, CI_{95} 13.00-21.74 %) and *L. monocytogenes* from 68/750 samples (9.07 %, CI_{95} 7.21-11.33 %).

The highest percentage of isolates of *Salmonella* spp. and *L. monocytogenes* were detected in tonsils (47.42 % of *Salmonella* isolates, 30.67 % prevalence in tonsils, CI_{95} 23.85-38.44 %; and, 86.76 % of *L. monocytogenes* isolates, 39.33 % prevalence in tonsils, CI_{95} 31.87-47.32 %, respectively). *Campylobacter* spp. was more frequently detected in faeces (83.49 % of isolates, 57.33% prevalence in faeces, CI_{95} 49.33-64.96 %) (Table 1).

The comparative statistical analysis between both slaughterhouses (A and B) showed a significant association with respect to *Salmonella* spp. isolation (χ^2 = 4.26; *P* = 0.039), with a higher prevalence of *Salmonella* spp. observed in slaughterhouse B. Thus, the probability of isolating *Salmonella* spp. was between 1.02 and 2.17 times higher in the slaughterhouse B (prevalence ratio = 1.49), which points out to this slaughterhouse as a risk factor. Not statistical association was detected between both slaughterhouses and *Campylobacter* spp. or *L. monocytogenes* isolation neither among pathogens themselves.

Table 1. Number of positive/tested samples and prevalence (%) with 95% confidence intervals (CI_{95}) of *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes* in each slaughterhouse (A, B) and in total (A+B). The highest prevalence on a pathogen basis is highlighted in bold.

Sample/slaughterhouse	*Salmonella* spp.		*Campylobacter* spp.		*Listeria monocytogenes*	
	Positive/tested	Prevalence % (CI_{95})	Positive/tested	Prevalence % (CI_{95})	Positive/tested	Prevalence % (CI_{95})
Skin (abrasive sponges)						
A	2/75		ND		0/75	
B	17/75		ND		2/75	
A+B	19/150	12.67 (8.26-18.93)	ND	ND	2/150	1.33 (0.36-4.72)
Ileocecal lymph nodes						
A	4/75		3/75		0/75	
B	8/75		4/75		4/75	
A+B	12/150	8.00 (3.66-12.48)	7/150	4.67 (2.27-9.31)	4/150	2.67 (1.04-6.65)
Faeces						
A	11/75		45/75		0/75	
B	6/75		41/75		0/75	
A+B	17/150	11.33 (6.22-16.42)	86/150	**57.33** (49.33-64.96)	0/150	0.00
Tonsils						
A	22/75		1/75		31/75	
B	24/75		1/75		28/75	
A+B	46/150	**30.67** (23.85-38.44)	2/150	1.33 (0.36-4.72)	59/150	**39.33** (31.87-47.32)
Meat						
A	0/75		0/75		0/75	
B	3/75		8/75		3/75	
A+B	3/150	2.00 (0.68-5.71)	8/150	5.33 (2.72-10.17)	3/150	2.00 (0.68-5.71)
TOTAL	97/750	12.93 (10.72-15.52)	103/600	17.17 (13.00-21.74)	68/750	9.07 (7.21-11.33)

On the other hand, the comparative study at the different stages of the production chain among slaughterhouses showed a statistically significant association ($\chi^2 = 13.57$; $P = 0.0002$) between the variable slaughterhouse and *Salmonella* spp. isolation from skin samples. In this case, the probability of isolating *Salmonella* spp. from skin samples was between 2.71 and 26.65 times higher in slaughterhouse B than in A (prevalence ratio = 8.50), which confirms slaughterhouse B as a risk factor. No statistical association was detected at other stages of the production chain among slaughterhouses. Whereas none of the selected pathogens could be isolated from meat samples in the slaughterhouse A, a low number of all the evaluated microorganisms were recovered from the slaughterhouse B (Table 1); however, no significant association was detected at this point.

2.1. *Salmonella* spp., *Campylobacter* spp. and *Listeria monocytogenes* typing and identification

From 97 *Salmonella* isolates, 13 different serotypes were identified (Table 2): monophasic *Salmonella* Typhimurium (mST) (n=21), Anatum (n=20), Typhimurium (n=17), Hessarek (n=15), Derby (n=8), Newport (n=7), Kentucky (n=3), Bredeney (n=1), Infantis (n=1), Rissen (n=1), Veneziana (n=1), Rubislaw (n=1) and Heidelberg (n=1). *Salmonella* Typhimurium phage types 104B (n=2), 104L (n=2), 193 (n=11), U302 (n=1) and U311 (n=1) were identified, with the following phage types identified from mST: 104B (n=1), 104L (n=2), 120 (n=1), 138 (n=2), 193 (n=6), U302 (n=7), U311 (n=1) and one nontypeable isolate.

Table 2. *Salmonella* spp. serotypes isolated at different sampling points in each slaughterhouse (A, B)

Sample/slaughterhouse	Positive/total isolates (n)	Serotypes (n; *S.* Typhimurium phage types)
Skin (abrasive sponges)		
A	2/97	Typhimurium monophasic (n=1; 104B); Typhimurium (n=1; 104B)
B	17/97	Anatum (n=14); Derby (n=2); Bredeney (n=1)
Ileocecal lymph nodes		
A	4/97	Typhimurium monophasic (n=2; 104L); Typhimurium (n=2; 104L)
B	8/97	Newport (n=4); Derby (n=3); Typhimurium monophasic (n=1; nontypeable)
Faeces		
A	11/97	Typhimurium monophasic (n=4; 193: 1, 138: 2, U302: 1); Hessarek (n=3); Kentucky (n=2); Typhimurium (n=2; US02: 1, 104B: 1)
B	6/97	Typhimurium monophasic (n=2; 193: 1, U311: 1); Derby (n=2); Newport (n=1); Infantis (n=1)
Tonsils		
A	22/97	Hessarek (n=12); Typhimurium monophasic (n=7; U302: 6, 120: 1); Kentucky (n=1); Rissen (n=1); Newport (n=1)
B	24/97	Typhimurium (n=11; 193:10, US311: 1); Anatum (n=6); Typhimurium monophasic (n=2; 193); Derby (n=1); Newport (n=1); Veneziana (n=1); Rubislaw (n=1); Heidelberg (n=1)
Meat		
A	0/97	-
B	3/97	Typhimurium monophasic (n=2; 193); Typhimurium (n=1; 193)

Seventy-three (73/103; 70.87 %) isolates were identified as *C. coli*, 3 (3/103; 2.91 %) isolates as *C. jejuni* and the remaining 27 isolates (27/103; 26.21 %) were confirmed as *Campylobacter* spp. (Table 3). Only two out of eight *Campylobacter* isolates recovered from meat samples corresponded to *C. coli* (Table 3).

Table 3. *Campylobacter* species identification at different sampling points in each slaughterhouse (A, B).

Sample/slaughterhouse	Positive/total isolates (n)	Species identified (n)
Ileocecal lymph nodes		
A	3/103	*C. coli* (n=2); *C. jejuni* (n=1)
B	4/103	*C. coli* (n=4)
Faeces		
A	45/103	*C. coli* (n=30); *C. jejuni* (n=2); *C.* spp. (n=13)
B	41/103	*C. coli* (n=34); *C.* spp. (n=7)
Tonsils		
A	1/103	*C. coli* (n=1)
B	1/103	*C.* spp. (n=1)
Meat		
A	0/103	-
B	8/103	*C. coli* (n=2); *C.* spp. (n=6)

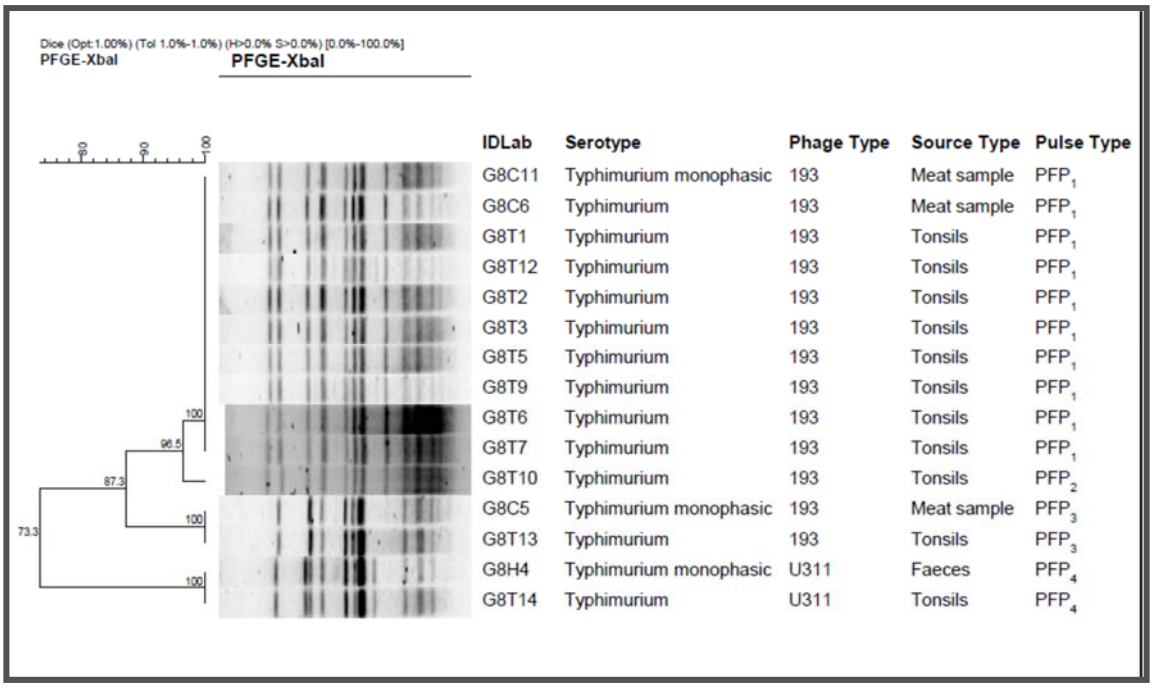
A total of 68 *L. monocytogenes* isolates were recovered with 39 isolates identified as serotype 4b, 28 isolates identified as serotype 1/2a and 1 nontypeable isolate obtained from an abrasive sponge. Isolates belonging to serotype 4b were obtained from abrasive sponges, ileocecal lymph nodes and tonsils; however, the three isolates recovered from meat samples belonged to serotype 1/2a (Table 4).

Table 4 . *Listeria monocytogenes* serotypes isolated at different sampling points in each slaughterhouse (A, B).

Sample/slaughterhouse	Positive/total isolates (n)	Serotypes (n)
Skin (abrasive sponges)		
A	0/68	-
B	2/68	4b (n=1); nontypeable (n=1)
Ileocecal lymph nodes		
A	0/68	-
B	4/68	4b (n=4)
Faeces		
A	0/68	-
B	0/68	-
Tonsils		
A	31/68	1/2a (n=13); 4b (n=18)
B	28/68	1/2a (n=12); 4b (n=16)
Meat		
A	0/68	-
B	3/68	1/2a (n=3)

2.2. PFGE and phylogenetic analysis.

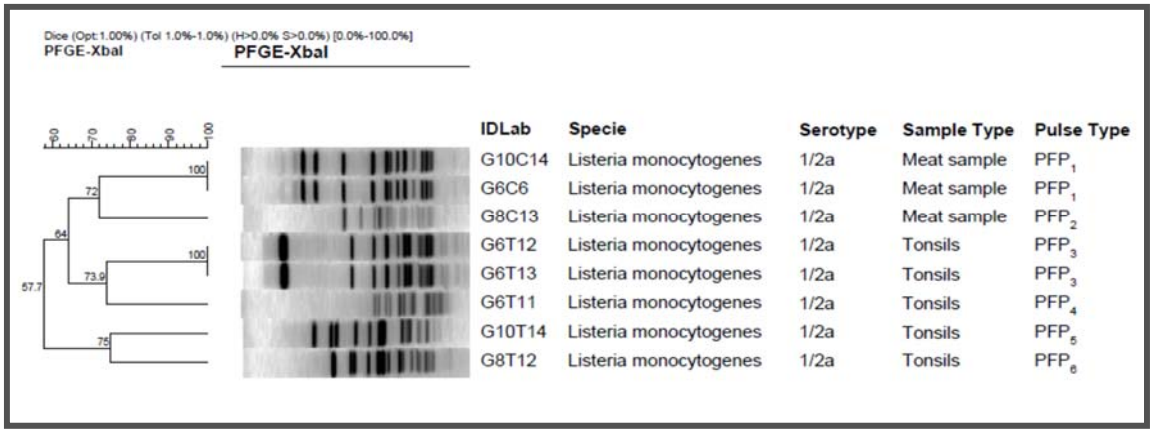
Four different PFGE patterns (PFPs) of *Salmonella* (including both *S.* Typhimurium and mST) with a genetic similarity coefficient value range of 73.30-100 % were identified in a same sampling (IDlab G8), in which the same clone was detected from meat and tonsils samples (Figure 1): $PFP_{1,}$ with eight isolates from tonsils (G8T1, T2, T3, T5, T6, T7, T9 and T11) and two isolates from meat samples (G8C6 and C11; 1 *S.* Typhimurium and 1 mST, respectively); PFP_2, with one strain from a tonsil; PFP_3 with one strain from meat (G8C5) and one from a tonsil (G8T13); and, PFP_4 with two isolates from the same pig, one isolate from faeces (G8H14) and the other one from the tonsils (G8T14).



IDLab	Serotype	Phage Type	Source Type	Pulse Type
G8C11	Typhimurium monophasic	193	Meat sample	PFP_1
G8C6	Typhimurium	193	Meat sample	PFP_1
G8T1	Typhimurium	193	Tonsils	PFP_1
G8T12	Typhimurium	193	Tonsils	PFP_1
G8T2	Typhimurium	193	Tonsils	PFP_1
G8T3	Typhimurium	193	Tonsils	PFP_1
G8T5	Typhimurium	193	Tonsils	PFP_1
G8T9	Typhimurium	193	Tonsils	PFP_1
G8T6	Typhimurium	193	Tonsils	PFP_1
G8T7	Typhimurium	193	Tonsils	PFP_1
G8T10	Typhimurium	193	Tonsils	PFP_2
G8C5	Typhimurium monophasic	193	Meat sample	PFP_3
G8T13	Typhimurium	193	Tonsils	PFP_3
G8H4	Typhimurium monophasic	U311	Faeces	PFP_4
G8T14	Typhimurium	U311	Tonsils	PFP_4

Fig 1. Dendrogram and Pulsed-Field Gel Electrophoresis (PFGE) profiles (PFPs) of *S.* Typhimurium showing genetic similarity index among isolates from different source samples of this study

PFGE-*Bsp*120I showed 6 different PFPs of *L. monocytogenes* serotype 1/2a obtained from three different days of slaughter from the same slaughterhouse (IDlab G6, G8 and G10), with a genetic similarity coefficient value ranging from 57.7 to 100 %: PFP_1, with two isolates obtained from meat samples from different days of slaughter (G6C14 and G10C14); PFP_2, with one strain from meat (G8C13); PFP_3, with two strains identified from tonsils (G6T12 and T13); and PFP_4, PFP_5 and PFP_6, with one strain from tonsils each (G6T11, G10T14 and G8T12, respectively) (Figure 2).



IDLab	Specie	Serotype	Sample Type	Pulse Type
G10C14	Listeria monocytogenes	1/2a	Meat sample	PFP_1
G6C6	Listeria monocytogenes	1/2a	Meat sample	PFP_1
G8C13	Listeria monocytogenes	1/2a	Meat sample	PFP_2
G6T12	Listeria monocytogenes	1/2a	Tonsils	PFP_3
G6T13	Listeria monocytogenes	1/2a	Tonsils	PFP_3
G6T11	Listeria monocytogenes	1/2a	Tonsils	PFP_4
G10T14	Listeria monocytogenes	1/2a	Tonsils	PFP_5
G8T12	Listeria monocytogenes	1/2a	Tonsils	PFP_6

Fig 2. Dendrogram and PFGE profiles (PFPs) of *L. monocytogenes* showing genetic similarity index among isolates from different source samples of this study

4. **Discussion**

The microbiological safety must be guaranteed at all levels of the food chain, with *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes* being considered as important food-borne pathogens, responsible for a large number of cases of disease in humans (EFSA, 2017). The goal of this study was to determine the prevalence and diversity of these pathogens along the slaughter line in two free-range pig slaughterhouses highlighting differences among them as critical control points to reduce pork carcasses contamination.

Currently, the most common perspective on food safety is the stable-to-table concept, acknowledging that each point in the food production chain has a responsibility of reducing the risk of foodborne diseases transmission (Davies and Funk, 1999).

Depending on many influential factors that include the herd status on the farm, transportation (length, stress factors, cleaning and disinfection procedures of trucks) and lairage conditions (waiting time, stress factors, separation of batches or cleaning and disinfection protocols), the microbiological status of the pigs entering the slaughter line may vary considerably between days or even batches (Lo Fo Wong et al., 2002). After a pig has entered the slaughter process, the final microbiological contamination of the carcass originates from one or more of the following sources: i) the animal itself; ii) previously slaughtered pigs via the machinery or personnel; or, iii) the environment or persistently contaminated equipment. These critical control points should be identified for each slaughterhouse and monitored as part of a Hazard Analysis Critical Control Point (HACCPP) system for contamination of the slaughter line (Lo Fo Wong et al., 2002).

In a previous study carried out by our research group we analysed the prevalence and characterization of *Salmonella* spp. in the slaughterhouse A evaluated in the present study (Hernández et al., 2013). Herein a reduction in the prevalence of *Salmonella* spp. at all stages of the production chain was observed in that slaughterhouse, with no *Salmonella* being recovered from meat samples after the intensification of the disinfection protocols applied at quartering as explained above. Slaughterhouse sampling results should always be carefully interpreted, depending on the type of sample collected and particular situations along the pork production chain (Swanenburg et al., 2001b). Although variations on the percentages of pre-slaughter positive samples (skin, tonsils, lymph nodes or faeces) should not be linked to changes in slaughter line protocols, these samples may reflect either the status and bacterial load of the animals at the farm or an early exposure of the animals during the transportation or at the lairage. In this sense, we hypothesise that bacterial load in these samples may be associated to the differences observed in meat positive samples among the two slaughterhouses analysed in the present study. This fact is supported by the higher probability of *Salmonella* isolation detected in our study in the slaughterhouse B when compared with slaughterhouse A, as well as with the greater *Salmonella* pre-slaughter exposure observed according to the statistical differences observed in skin samples. Furthermore, we also considered that differences between meat positive samples from both slaughterhouses (A and B) can be also attributed to more thorough cleaning strategies and sanitation processes implemented at slaughterhouse A, including the intensification of the disinfection protocols, and the routine sealing of the rectum before gut evisceration (Nesbakken et al., 1994) which were not performed in slaughterhouse B. In any case, when compared with previous reports from slaughter pigs reared in intensive systems (Swanenburg et al., 2001a; Argüello et al., 2013) a similar limited pork *Salmonella* contamination was observed in this study, even when high *Salmonella* risk farms were included in our study (individual seroprevalence ranging from 80 to 100%), which has

been described as a factor associated with increased *Salmonella* prevalence on pork samples (Swanenburg et al., 2001a).

In addition, it should be noted that a lower *Salmonella* prevalence was observed in pre-slaughter indicative samples of our study when compared with results of seropositive intensive farms from Swanenburg and coauthors (2001a). Despite it is common that a high *Salmonella* seroprevalence does not always correspond with high *Salmonella* prevalence, for instance, in faeces (Argüello et al., 2014), improved protocols in transportation or lairage procedures (reduced stress, length or improved disinfection protocols) have been reported as useful measures to control the transmission of *Salmonella* and other zoonotic agents before the arrival to the slaughter line in unconfined pig production systems (Lo Fo Wong et al., 2002).

The most prevalent serotypes isolated in the present study were mST, *S*. Anatum and *S*. Typhimurium, with both mST and *S*. Typhimurium isolated from all positive sources within this study. The high *S.* Typhimurium prevalence obtained in this work is in agreement with the last reports from EU isolates reported in pigs and pork samples, including both mST and *S*. Typhimurium (61.9 % and 26.9 %, respectively) (EFSA, 2017). In addition, a high diversity of mST and *S*. Typhimurium phage types was identified in this study, including those previously reported in free-range pigs (Gómez-Laguna et al., 2011) and pigs reared in intensive settings (Kirchner et al., 2011). Interestingly, the phage type 193, which is frequently isolated in human salmonellosis, was identified in meat samples from this study.

PFGE-*Xba*I results of this study showed evidences of *Salmonella* cross-contamination along different stages of the pork production chain: indistinguishable PFP_s were identified between tonsils from pigs of the

same herd, pointing to the exposure to a common pre-slaughter source of infection (on the farm, during transport or in lairage), and between tonsils and meat samples from different pigs of the same herd that were slaughtered the same day, pointing to a potential contamination during slaughtering and quartering (Swanenburg et al., 2001b).

In this study, *Campylobacter* was the bacteria most commonly isolated from the total of analysed samples followed by *Salmonella*. *Campylobacter coli* was the species most prevalent, followed by *Campylobacter* spp., and *C. jejuni,* which is consistent with previous reports (EFSA, 2017). In meat samples *Campylobacter* was the most ubiquitous pathogen in slaughterhouse B, whereas it was no detected in meat samples from slaughterhouse A. Taking into account the high *Campylobacter* prevalence obtained from pig faeces in this study, as previously mentioned, the sealing of the rectum performed in the slaughterhouse A may play a role in faecal contamination of pork with *Campylobacter* (Fosse et al., 2009). Most isolates recovered from meat samples were identified as *Campylobacter* genus, with two isolates being identified as *C. coli*. In this sense, it is noteworthy that the majority of fastidious *Campylobacteraceae* isolates obtained from porcine samples in a previous study contained virulence genes and antibiotic resistance indicating potential public health significance (Scanlon et al., 2013). These results highlight the necessity of implementing control measures along the slaughter line to avoid carcass contamination from faeces.

A similar prevalence of *L. monocytogenes* was observed in our study when compared with previous studies conducted in pig slaughterhouses (Fredriksson-Ahomaa et al., 2009; Hellström et al., 2010; Meloni et al., 2013). Despite the high prevalence of this pathogen in tonsils, a low contamination of meat samples was detected in our study. These results are in agreement with those previously reported by López et al. (2008), who could not isolate *L. monocytogenes* from free-

range pig carcasses. However, this microorganism was isolated from unfinished and finished meat products and environmental samples in this study, suggesting that persistent strains adapted to the processing plant could be involved (López et al., 2008). These results are in agreement with the PFGE results obtained in our study, in which indistinguishable PFPs of *L. monocytogenes* were isolated from pigs from different farms that were slaughtered in the same abattoir on different days, pointing to an environmental reservoir along the slaughter line as the most probable origin of the *L. monocytogenes* contamination detected on meat samples.

Listeria monocytogenes serotyping results of the present work are in accord with previous studies accomplished in pork processing plants, in which a high rate of serotype 1/2a was isolated (López et al., 2008; Hellström et al., 2010; Meloni et al., 2013); however, serotype 4b was the most frequent one in this study. Isolates of serotype 4b (lineage I) have obtained a special relevance for being overrepresented among human clinical isolates when compared to the serotype 1/2a (linage II), which is overrepresented among food and food-related as well as natural environments (Orsi et al., 2011). Interestingly, all *L. monocytogenes* strains isolated in the presented study belonged to serotypes 1/2a and 4b, with the three *L. monocytogenes* strains isolated from meat samples belonging to serotype 1/2a, which suggest the necessity of implementing control measures against this pathogen in free-range pig farms, slaughterhouses and processing plants.

5. Conclusions

Results of this study highlight the importance of implementing hygiene and sanitation strategies along the pork production chain to prevent pig carcass contamination by food-borne pathogens. In this sense, both pre-slaughter stages (on the farm, transport or lairage) and procedures during slaughtering and quartering, including the sealing of the rectum before gut evisceration and hydroalcoholic disinfection

protocols, were identified as critical control strategies to reduce pork carcass contamination by *Salmonella* spp., *Campylobacter* spp., and *L. monocytogenes*. In summary, our results highlight a low risk of free-range pork contamination by *Salmonella* spp., *Campylobacter* spp., and *L. monocytogenes* when hygienic and sanitation control measures along the pork production chain are implemented.

Acknowledgements

This study was financially supported by the Agency for Innovation and Development of Andalucía (IDEA; project references 351504/550859). Gómez-Laguna J is supported by a "Ramón y Cajal" contract of the Spanish Ministry of Economy and Competitiveness (RYC-2014-16735). Cardoso-Toset F was funded by a grant of the Agrifood Campus of International Excellence Programme (ceiA3) from the Ministry of Education, Culture and Sport and by the Santander Universities Global Division.

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Estudio 2 / Study 2

Estudio 2: "Effect of the probiotic Pediococcus acidilactici MA18/5M strain in reducing faecal *Campylobacter* spp. counts in free-range finishing pigs prior to slaughter"

Morales-Partera et al., 2019, under review

Objetivo específico 2: Identificar los puntos críticos de control y aplicar medidas correctoras que permitan disminuir la prevalencia de los distintos agentes zoonósicos detectados por su relación con la prevalencia de agentes zoonósicos alimentarios.

Effect of the probiotic Pediococcus acidilactici MA18/5M strain in reducing faecal Campylobacter spp. counts in free-range finishing pigs prior to slaughter

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Abstract

Pigs can be asymptomatic carriers of food-borne bacteria, such as *Salmonella enterica* and *Campylobacter* spp., which can pose a risk to human health. New strategies to control bacteria burden before reaching the slaughterhouse are necessary. This study evaluated the effect of *Pediococcus acidilactici* as alternative to antibiotics and its impact on performance parameters and faecal microbiota of finishing pigs. A total of 57 free-range pigs were randomly allocated and blocked by weight into two groups: (1) control group, with an initial BW of 143.28±14.26 kg which received a control diet; and (2) treated group, with an initial BW of 146.41±13.71 kg which received the control diet supplemented with *Pediococcus acidilactici* during the last 31 days of the finishing stage prior to their delivery to the slaughterhouse. At days 0, 22 and 31 from the beginning of the study weight and average daily gain (ADG) were recorded. Individual faecal samples from 8 pigs per group at 0 and 31 days were analysed for enumeration of *Enterobacteria, Escherichia coli, Campylobacter* spp., lactic acid bacteria and detection of *Salmonella* spp. A significant decrease in the counts of *Campylobacter* spp. in treated animals (P=0.004) in comparison with the control group was detected. This study indicates that supplementation with *Pediococcus acidilactici* may represent a useful approach to control *Campylobacter* spp. load in the intestinal tract of free-range finishing pigs prior to slaughter.

Keywords: *Campylobacter* spp.; free-range finishing pigs; faecal microbiota; *Pediococcus acidilactici*; performance.

1. Introduction

Pigs can be asymptomatic carriers of enterobacteria, such as *Salmonella enterica* and *Campylobacter* spp., acting as a source of contamination for other pigs and pork meat at the slaughterhouse (Argüello et al., 2013; Hernández et al., 2013; Morales-Partera et al., 2018). The control of pathogenic bacteria at farm as well as the use of adequate decontamination treatments during slaughter and dressing operations have been identified as critical stages for reducing the bacterial load of meat (Buncic & Sofos, 2012; Hernández et al. 2013; Morales-Partera et al., 2018).

During the last decades there has been an extended use of antibiotics at prophylactic doses as growth promoters to achieve high levels of efficiency in swine production (Davies, 2011; Nisha, 2008). This practice represents a risk to public and animal health, due to the potential persistence of residues in food products and in the environment, as well as the reduction of the effectiveness of antimicrobials against resistant bacteria (Abreu & Barreto, 2012). Nowadays, there is a growing concern for developing new feed additives as alternative to antimicrobials commonly used as growth promoters.

Lactic acid bacteria (LAB) can be used as growth promoters in swine (Quinto et al., 2014). Numerous beneficial effects of LAB upon swine, such as regulation of the intestinal microflora, inhibition of pathogens proliferation in the gastrointestinal tract, enhancement of the intestinal mucosal immunity and maintaining of the intestinal barrier function have been reported (Goktepe et al., 2005). Two parameters are thought to be important for selection of LAB as probiotics. First, the LAB must be able to survive the upper gastrointestinal tract showing a high resistance to inorganic acids (i.e. hydrochloric acid), bile salts and pancreatic enzymes (Pringsulaka et al., 2015; Shin et al., 2014). Second, LAB must adhere to the intestinal epithelial cells (Kadlec & Jakubec, 2014; Lähteinen et al., 2010), which favour bacterial colonization, pathogen exclusion and host cell interactions (Lebeer et al., 2008). In this sense, *Pediococcus acidilactici* has been pointed as a candidate of interest as probiotic in pigs (Balgir et al., 2013; Barbosa et al., 2015; Brashears et al., 2003; Fernández et al., 2014; Kiran et al., 2015; Lessard et al., 2009; Todorov et al., 2011). *Pediococcus* competes for binding sites (Ganan et al., 2012) and produce pediocin which has

broad spectrum activity against bacteria, such as *Listeria*, *Clostridium*, *Bacillus*, *Aeromonas*, *Staphylocoocus*, *Lactobacillus* and *Enterococcus* (Niamah, 2018).

In vitro studies have confirmed the functional and safety properties of *Pediococcus acidilactici* and highlight the need for *in vivo* studies (Sirichokchatchawan et al., 2018). Thus, the implementation of new control strategies at the slaughterhouse to limit the bacterial load of carcasses is encouraged. The present study was addressed to evaluate the effect of *Pediococcus acidilactici* supplementation on performance parameters and faecal flora in finishing pigs.

2. Materials and Methods

2.1. Animals and experimental design

This experiment and all animal procedures were performed according to the guidelines of the European Union (Directive 2010/63/EU) and approved by the local ethics committee. A total of fifty-seven 10 to 12 months old Iberian, free-range pigs were randomly allocated and blocked by weight into two groups: (1) control group (n = 29), with an initial BW of 143.28±14.26 kg which received control diet along the study; and (2) treated group (n = 28), with an initial BW of 146.41±13.71 kg which received the control diet supplemented with *Pediococcus acidilactici* MA18/5M during the last 31 days of the finishing stage prior to their delivery to the slaughterhouse. Both control and treated groups received conventional feeding for finishing of Iberian pigs (Table 1).

Thirty-one days before slaughter (day 0 of the study) treated animals were supplemented with a commercial additive containing *Pediococcus acidilactici* MA18/5M (Bactocell, Lallemand, Blagnac Cedex, France) at a rate of 200 g/T, according to manufacturer's instructions, reaching a final average concentration of 1.8×10^4 CFU/g. In addition, the drinking water in the lairage at the slaughterhouse was also supplemented with the same commercial additive containing *Pediococcus acidilactici* MA18/5M (Bactocell soupe, Lallemand) at a rate of 200g/1000L according to manufacturer's instructions, reaching an average final concentration of 8.4×10^4 CFU/L. All animals were daily monitored for the presence of any clinical sign of disease. At days 0, 22 and 31 all the pigs from each experimental group were weighed

and the weight evolution and average daily gain (ADG) were recorded. Data are expressed as the mean ± SD of the animals per treatment (control *vs*. treatment).

Table 1. Composition and chemical analysis of the basal diet given to free-range pigs in both groups along the study.

Ingredients Composition	**g/Kg**
Barley grain	574.60
Corn grain	19.20
Lard	3.50
Calcium Carbonate	19.93
NaCl	3.00
Rapeseed meal	50.90
Golden Corn DDGS[1]	11.80
Decorticated sunflower meal	60.00
Wheat Gluten Feed	80.00
Wheat Bran	120.00
Malt culms	20.00
Soybean hull	30.00
Organic acids	1.00
Pre-mix	6.00
Nutrient Composition	g/Kg
Crude Protein	141.00
Lysine	6.97
Methionine	2.70
Methionine + Cystine	5.92
Threonine	5.40
Tryptophan	1.75
Total lipids	25.58
ME[2], MJ/Kg	12.34
Digestible Protein/ME, g/MJ	9.14

[1]DDGS: Distillers Dried Grains Solubles; [2]ME: Metabolic Energy

2.2. Faecal microbiota analysis

Individual faecal samples were aseptically obtained directly from rectum of 8 pigs from each group at days 0 and 31. All samples were collected into sterile containers and were transported to the laboratory and analysed on the same day of collection. Ten-fold serial dilutions of each sample were inoculated onto selective medium by using specific ISO methodologies for enumeration of *Enterobacteriaceae* (ISO 21528:2.2017), *Escherichia coli* (ISO 16649:2015)*, Campylobacter* spp. (ISO 10272:2006), LAB (ISO 15214:1998) and ISO methodologies for detection of *Salmonella* spp. (ISO 6579:2002).

Results are expressed as logarithmic colony forming unit per gram of faeces (log CFU/g) for *Enterobacteriaceae*, *Escherichia coli*, *Campylobacter* spp. and LAB. For *Salmonella*, presence or absence was recorded.

2.3. Statistical analysis

To carry out the statistical analysis of the different parameters, the normal distribution of data was assessed by means of D'Agostino and Pearson omnibus normality test (GraphPad Prism v.5.0, GraphPad Software, San Diego, California, USA). When data presented a normal distribution, the differences between the mean of the examined parameters were assessed by means of a simple ANOVA test followed by a paired or unpaired t-test, as appropriate. For data without a normal distribution, the differences between the mean of the examined parameters were assessed by means of Kruskal Wallis test followed by a Mann Whitney test. All variables were analysed with GraphPad Prism v.5.0. Significant differences were considered at P values < 0.05.

3. Results

3.1. Performance parameters

No clinical signs of disease were detected in any animal belonging to both control and treated groups along the study. Table 2 shows the weight and the average daily gain throughout the study of the animals included in each group. No differences were detected between the initial or final body weight of animals belonging to treated or control groups (P > 0.05). In addition, no differences were observed in ADG values among groups (P > 0.05) (Table 2).

Table 2. Performance characteristics (mean ± standard deviation) of pigs fed with a basal diet (control group) or supplemented with *Pediococcus acidilactici* MA18/5M (treated group) during the last 31 days of the finishing period.

	From day 0 to day 22			From day 22 to day 31			From day 0 to day 31		
	Control group	**Treated group**	***P***	**Control group**	**Treated group**	***P***	**Control group**	**Treated group**	***P***
Initial body weight (kg BW)	143.28±14.26	146.41±13.71	NS	164.16±10.29	168.15±8.66	NS	143.28±14.26	146.41±13.71	NS
Final body weight (kg BW)	164.16±10.29	168.15±8.66	NS	171.12±10.26	174.69±8.10	NS	171.12±10.26	174.69±8.10	NS
ADG (kg)*	0.912±0.178	0.976±0.246	NS	0.987±0.375	0.891±0.548	NS	0.898±0.161	0.901±0.173	NS

*ADG: Average Daily Gain

3.2. Intestinal microbiota

Table 3 summarizes the results of the microbiological analyses from faeces of both experimental groups. No differences were observed in the counts of the selected microorganisms from animals belonging to the control group between day 0 and 31 ($P > 0.05$). On the other hand, similar counts were obtained of enterobacteria, *Escherichia coli* and lactic acid bacteria between day 0 and 31 in the treated group. However, a decrease ($P = 0.004$) in the counts of *Campylobacter* spp. (4.86-3.40 log CFU/g), with an approximately 1.5 log CFU/g lower bacterial load at the end of the study, was obtained. *Salmonella* spp. was detected neither in control nor in treated animals at day 0 or 31.

Table 3. Counts (log CFU/g) of selected bacteria in the faeces of pigs fed with a basal diet (control group) or supplemented with *Pediococcus acidilactici* MA18/5M (treated group) during the last 31 days of the finishing period[1].

	Control Group			**Treated Group**		
	Day 0	**Day 31**	***P***	**Day 0**	**Day 31**	***P***
Enterobacteria	6.25±0.53	5.94±0.92	NS	6.26±0.65	6.26±0.57	NS
Escherichia coli	5.98±0.81	5.63±1.09	NS	5.66±1.15	6.25±1.14	NS
***Campylobacter* spp.**	4.79±0.54	4.02±1.20	NS	4.86±0.34[a]	3.40±0.94[b]	0.004**
Lactic Acid Bacteria	8.89±0.29	8.94±0.46	NS	9.27±0.97	8.99±0.33	NS

[1]*Salmonella* spp. was absent both in control and in treated animals at day 0 or 31

4. Discussion

The food industry demands the development of new strategies that allow decreasing bacterial burden of animals at the slaughterhouse as well as increasing the quality of food products and the food safety. The use of lactic acid bacteria has been pointed out as an approach of interest by competing with pathogenic bacteria for colonization sites, promoting an antioxidant status and modulating the host immune response (Lebeer et al., 2008). Specifically, *Pediococcus acidilactici* has been showed to enhance carcass quality and physicochemical properties of pork highlighting its potential use as probiotic in finishing pigs (Dowarah et al., 2018). In the present study, the use of *P. acidilactici* at the finishing stage of free-range pigs was addressed to analyse its impact in performance parameters as well as on the intestinal microbiota one month prior to slaughter.

Supplementation with *Pediococcus acidilactici* has been previously used in nursery and weaning pigs as a probiotic of interest in reducing the use of antimicrobials as well as in the control of peri-weaning diarrhoea. Interestingly, these studies have evidenced a marked *in vitro* adhesion to pig intestinal epithelium cell (Dowarah et al., 2018), the stimulation of the innate immune defence *in situ* at ileum but not at systemic level (Daudelin et al., 2011; Lessard et al., 2009) as well as an increase in villi height and crypts depth in treated animals in comparison with controls (Di Giancamillo et al., 2008). Moreover, the addition of *P. acidilacti* has been showed to decrease ileal bacterial diversity and increased the Firmicutes proportion in pigs at 2 weeks post-weaning (Brousseau et al., 2015). All these findings support, at least in part, the rationale of using *P. acidilactici* as probiotic in pigs to improve gut health. Despite there are not homogeneous results regarding the impact of *P. acidilactici* on performance parameters in piglets (Di Giancamillo et al., 2008; Dowarah et al., 2018; Lessard et al., 2009), some studies have showed an improved feed conversion ratio without significant effect in average daily gain which might be associated to an apparent better digestibility of crude protein (Dowarah et al., 2018). In our study, no changes in performance parameters were observed in finishing pigs. The discrepancies observed between different studies may be linked to several factors such as animal genetics and breeds, diet composition or management practices among others.

The results of the faecal microbiota analysis showed a significant decrease in the log CFU/g of *Campylobacter* spp. in pigs fed with a diet supplemented with a commercial additive containing *Pediococcus acidilactici* MA18/5M. Our results suggest that the strain of *Pediococcus acidilactici* used in the present study is active against *Campylobacter* spp. This behaviour has been associated with a competition for nutrients in the gut, competition for binding sites on the intestinal epithelium (Malago et al., 2011) as well as the production of bacteriocins. The decrease in pH by LAB has been proved to have a limited effect upon *Campylobacter* spp. load (Brus et al., 2013). Experimental studies have showed the potential of *P. acidilactici* in reducing the attachment of *E. coli* harbouring the F4 (K88) fimbriae (ETEC F4) to the intestinal mucosa (Daudelin et al., 2011) as well as ETEC translocation to mesenteric lymph node (Lessard et al., 2009). However, no effect on *E. coli* counts was observed in our study. Different hypotheses can be suggested to explain this result. One

possible explanation is that the reduction in *Campylobacter* spp. counts may effectively reduce nutrient competition against *E. coli* (Korhonen & Martikainen, 1991). Another possibility is that *P. acidilactici* may successfully competes for binding sites with *E. coli* meaning less bacteria retained within the bowel and leading to more bacteria being expelled through faeces (Ouwehand & Conway, 1996). The lack of changes in the counts for *E. coli*, as well as for *Enterobacteriaceae*, observed in the present study may be also associated to the short period of supplementation in the present study, one month prior to the delivery to the slaughterhouse. Taking all this into account, further studies should be conducted with supplementation for longer periods to analyse its impact both on performance parameters and on faecal microbiota.

In both experimental groups no changes were observed in the counts of LAB. The lack of changes in the counts of LAB in *P. acidilactici* treated animals point out that either *P. acidilactici* could be displacing other LAB or that a more prolonged supplementation could be required to observe a sustained increase in LAB load.

5. Conclusions

The properties attributed to most of LAB are for the most part based upon results from *in vitro* conditions, which assumes empirical use in pigs without rigorously assess the effects in this species. The absence of enough field tests validating the properties of commercially available additives in livestock is evident. This study shows that free-range pigs fed with a diet supplemented with a commercial additive containing *Pediococcus acidilactici* MA18/5M did not induce changes on average daily gain but succeed in reducing the *Campylobacter* spp. load in the faeces of treated animals. Further studies should be conducted to evaluate the impact of *P. acidilactici* after prolonged supplementation at the finishing stage of pigs and prior to their delivery to the slaughterhouse.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

This study was financially supported by the Agency for Innovation and Development of Andalusia (IDEA; project references 351504/550859). J. Gómez-Laguna is supported by a "Ramón y Cajal" contract of the Spanish Ministry of Economy and Competitiveness (RYC-2014-16735). F. Cardoso-Toset was funded by a grant of the Agrifood Campus of International Excellence Programme (ceiA3) from the Ministry of Education, Culture and Sport and by the Santander Universities Global Division.

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Estudio 3 / Study 3

Estudio 3 “Viability of selected foodborne pathogens in dry cured pork loins”

Morales-Partera et al., 2017, Int J Food Microbiol, 258, 68-72

Objetivo 3: Determinar la viabilidad de patógenos seleccionados frente a las condiciones de curación empleadas en los productos del cerdo Ibérico:

Survival of selected foodborne pathogens on dry cured pork loins

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Abstract

The safety of ready-to-eat products such as cured pork loins must be guaranteed by the food industry. In the present study, the efficacy of the dry curing process of pork loins obtained from free-range pigs in the reduction of three of the most important foodborne pathogens is analysed. A total of 28 pork loin segments, with an average weight of 0.57 ± 0.12 kg, were divided into four groups with three being inoculated by immersion with 7 log CFU/ml of either *Salmonella* Typhimurium*, Campylobacter coli* or *Listeria innocua* and the last one inoculated by immersion with sterile medium (control group). The loin segments were treated with a seasoning mixture of curing agents and spices, packed in a synthetic sausage casing and cured for 64 days. Microbiological analysis, pH and water activity (a_w) were assessed at four stages. The values of pH and a_w decreased with curing time as expected. *S*. Typhimurium and *C. coli* dropped significantly (3.28 and 2.14 log units, respectively), but limited reduction of *L. innocua* (0.84 log unit) was observed along the curing process. In our study, three factors were considered critical: the initial concentration of the bacteria, the progressive reduction of pH and the reduction of a_w values. Our results encourage performing periodic analysis at different stages of the manufacturing of dry-cured pork loins to ensure the absence of the three evaluated foodborne pathogens.

Keywords: *Salmonella*; *Listeria*; *Campylobacter*; pork loins; dry-curing; water activity.

Introduction

The production of safe and healthy products represents one of the main objectives of the food industry worldwide. Food products continue to be responsible for important outbreaks of disease in consumers (Larsen et al., 2014). According to the latest data published by the European Food Safety Authority (EFSA), a total of 4,362 foodborne outbreaks were reported in 2015, with *Campylobacter* and *Salmonella* species being responsible for a significant percentage of these cases (EFSA, 2016). Another pathogen that has become more important due to its notable and extreme severity is *Listeria monocytogenes* (Álvarez-Fernández et al., 2012; Magalhães et al., 2014). In this scenario, meat products have been identified as significant sources of these foodborne pathogens (Gómez et al., 2014; Holley and Cordeiro, 2014).

Animals can be healthy carriers of these pathogens at different organic locations, which can be triggered during stressful situations, such as transport or slaughter procedures. Also, these pathogens can contaminate the meat from the animal's intestinal content or skin which may then be spread by utensils, machinery, water and food handlers and processors (Buncic and Sofos, 2012; Choi et al., 2013).

Dry-cured pork products obtained from pigs reared under traditional breeding systems, such as the Iberian pig, are highly appreciated by consumers. The characteristic flavour and high quality, in addition to traditional and environmentally and friendly production practices, are important reasons for the growing demand of dry-cured hams, shoulders and loins (Ruiz et al., 2002). The manufacture of these products includes the maturation of pork meat with different additives (salts, spices and other ingredients), and the subsequent dehydration and ripening procedures to obtain shelf stable ready-to-eat products (Soto et al., 2008).

Foodborne pathogens such as *Salmonella* spp., *L. monocytogenes* and *Campylobacter jejuni* have been shown to survive some fermentation, maturation and drying procedures necessary to obtain dried and fermented meat products (Lucke, 2009; Hong et al., 2016). Furthermore, some of these microorganisms have been detected in cured and fermented dried meats sampled from markets and specialty food shops (Gormley et al., 2009). Although previous studies have tested the viability of selected foodborne pathogens during the manufacturing of some dry-cured meat products (Reynolds et al., 2001; García-Díez et al., 2016), the efficacy of the manufacturing procedure of dry-cured Iberian pork loin to eliminate these microorganisms from contaminated fresh meat has not been previously evaluated.

For that reason, the objective of this work was to evaluate the survival of three pork associated foodborne pathogens, namely *Salmonella* Typhimurium, *Listeria monocytogenes* and *Campylobacter coli*, along the dry curing process of experimentally inoculated free-range Iberian pork loins.

Materials and Methods

Bacterial strains and inoculum preparation.

Monophasic *S.* Typhimurium 1,4,[5],12:i:- DT 193 and *C. coli* strains (one each) used in this study were recovered from pig faeces and identified by specific ISO methodologies (6579:2002 and 10272:2006, respectively). Serotyping of *Salmonella* was performed by means of an agglutination technique using commercially available antisera (Statens Serum Institut, Copenhagen, Denmark) and a phage's panel provided by the International Reference Laboratory of phage typing (Central Public Health Laboratory, London, UK) at the National Centre of Microbiology (Institute of Health Carlos III, Madrid, Spain). A multiplex PCR assay was performed to identify *Campylobacter* to species level (Yamazaki et al., 2008).

Listeria innocua serotype 6a CECT 910 (Spanish Type Culture Collection, Valencia, Spain), originally isolated from a cow brain (Seeliger H. 1979,

Wurzburg, Germany), was selected as a surrogate of *L. monocytogenes* to prevent unnecessary exposure to this pathogen during experimental processing (Friedly et al., 2008; Barbiroli et al., 2016).

All the strains were stored at -80 ºC in Brain Heart Infusion (BHI) broth (Oxoid, Madrid, Spain) containing 20 % glycerol (Scharlab, Barcelona, Spain) until use.

For inoculum preparation, strains were plated onto Trypticase soy agar (Oxoid) and incubated at 37 ºC for 24 hours in aerobiosis (*S.* Typhimurium and *L. innocua*), or at 42 ºC for 48 hours under microaerophilic atmosphere (*C. coli*), using a GENbagMicroaer (BioMérieux, Madrid, Spain). Afterwards, 4 to 5 colonies were isolated and resuspended in 5 ml of BHI medium (Oxoid), and incubated in the same conditions (Merialdi et al., 2015). Then, cultures were counted by using plate counts and absorbance spectrophotometer at a wavelength of 595 nm.

Subsequently, 0.1 ml of this culture were transferred to 9 ml of BHI medium (Oxoid) and incubated at the same conditions to obtain an inoculum of 10^8 CFU/ml of each pathogen that was adjusted to 10^7 CFU/ml by serial dilutions in 0.9 % NaCl and 0.1 % sterile peptone water solution (Oxoid). Bacterial counts were checked by plating on the same conditions in each assay.

Dry cured pork loins manufacturing, inoculation procedure and sampling

A total of 14 pork loins (*M. longissimus dorsi*) were obtained from freshly slaughtered Iberian free-range pigs after routine procedures in a commercial slaughterhouse. After butchering, loins were divided into two segments and a total of 28 pork loin segments with an average weight of 0.57 ± 0.12 kg were frozen at -20 ºC until analysis.

For the experiment, loins were allowed to defrost under refrigerated conditions at 4 ºC for 2 days. The segments were UV irradiated for 30 min in a laminar flow hood to reduce surface contamination (Keklik et al., 2010). A total of 24 loin segments were inoculated by immersion for 2 min with a concentration of 7 log CFU/ml of each microorganism in 0.9 % NaCl and 0.1 % sterile peptone water solution (Oxoid) (8 loins/pathogen) to obtain an initial bacterial load of approximately 5 log CFU/ml of each pathogen (Cardoso-Toset et al., 2017). Four additional loin segments were immersed in sterile peptone water solution (Oxoid) and used as controls. After immersion, loins were placed on plastic racks at room temperature (24 ºC) for 10 min to allow microbial attachment and stored at 4 °C for 24 hours. After chilling, each loin segment was irrigated with water for 15 seconds to select only superficially attached bacteria (Warriner et al., 2001). Then, a seasoning mixture of curing agents (salt, nitrates and nitrites) and spices (paprika from *Capsicum annuum* and powdered garlic from *Allium sativum*) was added in a ratio of 48 g/kg and macerated for 5 days in plastic vats at 3-4 °C and 85 % relative humidity (RH) to allow penetration into the meat (Cardoso-Toset et al., 2017). Following this, the loin segments were packed in a synthetic sausage casing made of collagen and stored at 6-7 ºC and 85 % RH for a week. Temperature was then increased to 8-10 ºC and RH was reduced to 75 % for 50 days. Finally, loins were maintained at 10-12 ºC and 75 % RH until the end of the curing process (64 days in total) (Fig. 2).

Inoculated and control loins were analysed at four different stages throughout the process: 24 hours post-inoculation (T1), at the post-additive stage (T2), after the first week of drying (T3) and at the final stage of the dry-curing process (T4) (Fig. 2). In each sampling, four pieces per pathogen (R1-R4) and two controls (C1-C2) were analysed.

Physicochemical analysis

Water activity (a_w), pH and weight loss values (expressed as percentage of the initial weight) of dry-cured loins were measured at the end of the experiments to evaluate the stability and quality of the final product. The evolution

of pH and a_w values of dry-cured loins was also evaluated during each processing stage. A pH meter (Crison, Barcelona, Spain) equipped with a solid electrode for penetration inside the loin (2 cm approximately) was used for pH analysis. Special care was taken to measure muscular tissue. A computer-based dew point method was used for the measurement of a_w (Aqualab, Pullman, USA).

2.4. Microbiological analysis

For enumeration, 10 g of loin samples with dimensions of 2 x 2 cm^2 from surface region were cut and homogenised with 90 ml of buffered peptone water (Oxoid) in a Stomacher® 80 (Seward Ltd., West Sussex, UK). This homogenate was serially diluted (1/10) in the same medium and 0.1 ml of appropriate dilutions were plated onto selective media.

In addition, to evaluate the possible presence of viable bacteria when the bacterial count was under the plate detection limit (<2 log CFU/g), a sample of 25 g of each loin was also individually homogenized with 225 ml of specific pre-enrichment broth during 90 seconds in a Stomacher® 80 (Seward Ltd.). Cultures were performed using specific ISO methodologies for the detection of *Salmonella* spp., *L. monocytogenes* and *Campylobacter* spp. (6579:2002, 11290:2000 and 10272:2006, respectively).

Data analysis

Results are presented as log value of CFU/g and expressed as mean ± standard deviation (SD). For statistical purposes, absence of pathogens in 10 g of product was considered to be zero log CFU/g and presence of pathogens in the enriched homogenate when counts were below to the plate detection limit (< 2 log CFU/g) were considered to be 1.95 log CFU/g (Stollewerk et al., 2012).

The values were evaluated for approximate normality of distribution by the D'Agostino & Pearson omnibus normality test (GraphPad Prims v5.0, USA).

When data did not follow a normal distribution, differences among the means of control and treated groups were assessed by Kruskal-Wallis test followed by Dunn's Multiple Comparison test (GraphPad Prims v5.0, USA). Comparison between two groups was performed with Mann Whitney-U non-parametric test or Wilcoxon signed rank test for non-parametric paired values (GraphPad Prims v5.0, USA). Comparison between two groups with a normal distribution of the data was assessed by paired-t test (GraphPad Prism v5.0 USA). Differences with a $P < 0.05$ were considered to be statistically significant.

Results

Physicochemical parameters (pH, a_w and weight loss) evaluated in dry-cured loins

The evolution of the physicochemical parameters evaluated along the dry-cured loins processing is showed in Table 1. A progressive reduction of pH and a_w was obtained from T1 to T4, with a final average pH value of 5.56 ± 0.12 and a final average a_w value of 0.887 ± 0.03 ($P < 0.05$; Table 1). As expected, the average weight loss value reached around 40% when dry-cured loins were fully processed.

Behaviour of selected foodborne pathogens during the dry-cured processing of pork loins

The average bacterial load of the three inoculated pathogens along the dry-cured loins processing is showed in Fig. 1. The results show a significant drop of *S*. Typhimurium ($P = 0.03$) over the curing period from 5.23 ± 0.53 log CFU/g in the initial phase to 1.95 log CFU/g (4 out of 4 replicates with viable bacteria under plate detection limit) in the final product which equated to a percentage reduction of 62.68 % (3.28 log CFU/g). A progressive reduction was observed in all tested stages (T1-T4), although it was not significant in the post-additive stage (T2; $P = 0.69$) and at the end of the first week of drying (T3; $P = 0.11$) (Fig. 1).

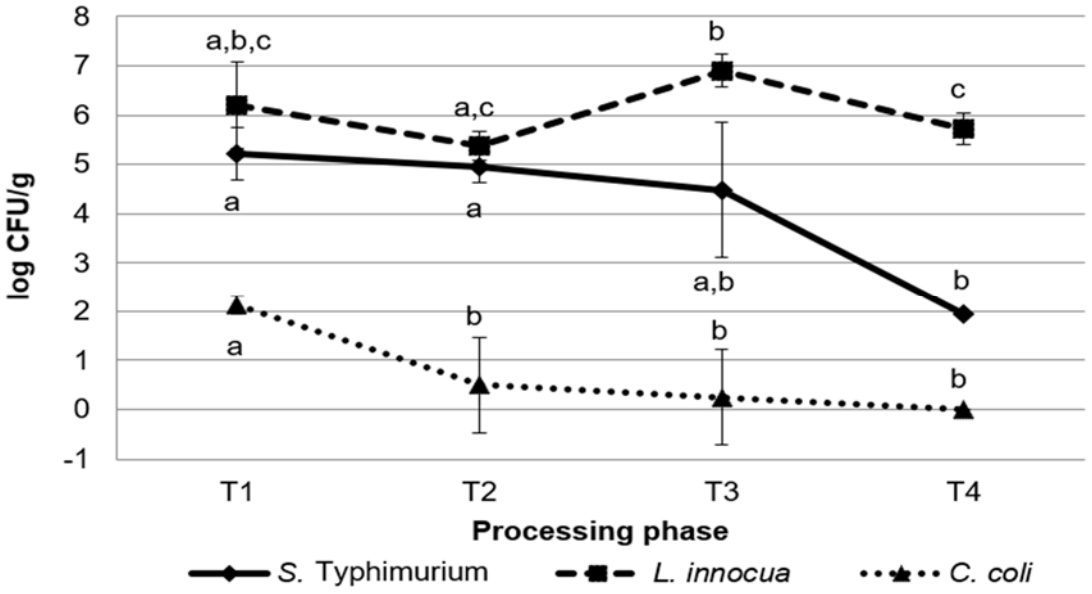

Fig. 1. S. Typhimurium, *L. innocua* and *C. coli* average bacterial load reduction along dry-cured loins processing. T1: 24-hour post inoculation. T2: post-additive. T3: end of the first week of drying. T4: final product.

L. innocua concentration value was 6.21 ± 0.88 log CFU/g at T1. The counts for *L. innocua* fluctuated between the different stages of analysis with an overall no significant reduction with respect to the initial stage (reduction of 7.69 %; 0.48 log CFU/g). In the additive stage (T2), the greatest reduction in pathogen concentration was observed with counts of 5.37 ± 0.29 log CFU/g, which represented a reduction of 13.53 % (0.84 log CFU/g) in relation to the initial stage (P = 0.34). A significant increase in *L. innocua* concentration was observed between T2 and T3 (P = 0.03), followed by a significant decrease between the end of the first week of drying (T3) and the final product (T4) (P = 0.03) (Fig. 1).

C. coli counts before the loins were macerated were of 2.14 ± 0.19 log CFU/g. This value was significantly reduced between T1 and T2 (P = 0.04). In the post-additive stage (T2) and at the end of the first week of drying (T3), the observed concentrations were 0.49 ± 0.98 log CFU/g and 0.25 ± 0.50 log CFU/g, respectively. The final product was assessed to be absent for *C. coli* indicating a 100 % reduction over the production process (P = 0.03) (Fig. 1).

No pathogens were detected in control samples at the different analysed stages by using the enumeration and detection methods.

Discussion

The microbiological safety of food products derived from animals must be guaranteed to ensure consumer confidence and to allow the exportation of products such as dry-cured ham or loins, which are highly appreciated by consumers worldwide. In this context, it is important to validate each specific process and evaluate each potential biological risk, as different mechanisms of inactivation may be operating (Lindqvist and Lindblad, 2009).

In this work, the behaviour of *S.* Typhimurium, *L. monocytogenes* (using *L. innocua* as a surrogate) and *C. coli,* three of the most important foodborne bacteria associated with pigs and pork products, were studied throughout the dry curing process of Iberian pork loins obtained from free-range pigs. For this purpose, a physicochemical analysis and a microbiological analysis (based on enumeration and detection studies) of Iberian pork loins inoculated with a known concentration of *S.* Typhimurium, *L. innocua* and *C. coli* were carried out at four stages: 24 hours post-inoculation (T1), after seasoning mixture addition (T2), one week after drying (T3) and at the final product (T4).

When physicochemical parameters were considered, the average pH and a_w values were similar to those found during the manufacturing process in this type of product (Ruiz-Ramírez et al., 2005; Lee et al., 2014; Cardoso-Toset et al., 2017).

A decrease in bacterial counts of the three evaluated microorganisms was obtained during the processing of pork loins; however, differences were detected between them. Whereas there was a progressive reduction of *S*. Typhimurium in

all tested phases (T1-T4), the behaviour of *L*. *innocua* fluctuated throughout the study. Despite *Listeria* counts dropped after the additive phase (T2), an increase of this parameter was observed at the end of the first week of drying (T3), as previously reported in hams (Reynolds et al., 2001). This result demonstrates the tolerance of this pathogen to the dry-curing process, which is probably related with its tolerance to salts and low temperatures in the presence of organic material (Magalhães et al., 2016), and highlights the importance of controlling *L. monocytogenes* throughout the processing of cured products. The bacterial count reduction observed at the end of the manufacturing process, may also be associated with the reduced pH and a_w values (Mataragas et al., 2015) which provide an unfavourable environment for the development of numerous pathogenic and food spoilage bacteria (O'Bryan et al., 2015).

Contrary to *Salmonella* and *Listeria*, low initial *C. coli* counts were recovered at the beginning of the study. The microaerophilic condition of this microorganism together with the inoculation procedure by immersion may have an impact on this finding. In the present study, a significant reduction (P = 0.04) in *C. coli* counts was observed after the post-additive stage (T2), result that suggests a greater sensitivity of this pathogen to additives compared to the other analysed microorganisms (Sampers et al., 2010). Nonetheless, the absence of this pathogen in the final product could also be related to the lower initial concentration of *Campylobacter* in our study.

According to the literature, the dry curing process generally inhibits the proliferation of foodborne pathogens due to the concurrence of several microbial hurdles, such as a low pH and a_w, a high salt concentration, the addition of nitrites, spices and other ingredients or the growth of competitive flora that contribute to the final product stability (Stollewerk et al., 2012). The results of the present study show that these bacteriostatic parameters are able to reduce the load of some foodborne pathogens, such as *S.* Typhimurium and *C. coli*, after processing of dry-cured Iberian pork loins. However, considering that the complete elimination of *S.* Typhimurium and *L. innocua* was not obtained, the initial concentration of

these pathogens on fresh loins may be considered as a critical point to obtain safe ready-to-eat dry cured loins. In this sense, the control of pathogenic bacteria at farm as well as the use of adequate decontamination treatments during slaughter and dressing operations have been identified as critical stages for reducing the bacterial load of the meat (Buncic and Sofos, 2012; Hernandez et al., 2013). These facts point out the importance of implementing strict controls on the presence of these pathogens in ready-to-eat dry cured loins (Commission Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs).

Although the results obtained in this study characterise the effectiveness of the dry curing process in reducing some pathogenic bacteria, such as *S*. Typhimurium and *C. coli*, future studies which include different strains of each pathogen would be valuable to rule out the possibility of intra-species variability in the susceptibility of the inoculated pathogens to the dry curing process.

Conclusion

In this study, logarithmic levels of *S.* Typhimurium and *C. coli* declined over the production process of dry cured pork loins, whereas only mild changes were observed for *L. innocua* counts. We identified three critical factors to justify our findings: (i) the initial concentration of the bacteria; (ii) the progressive reduction of pH; and (iii) the reduction of a_w values. Although a reduction of bacterial counts throughout the process is observed, the results of this study do not support that the production process of dry-cured pork loins completely removes *S.* Typhimurium and *Listeria monocytogenes* in case of high initial contamination.

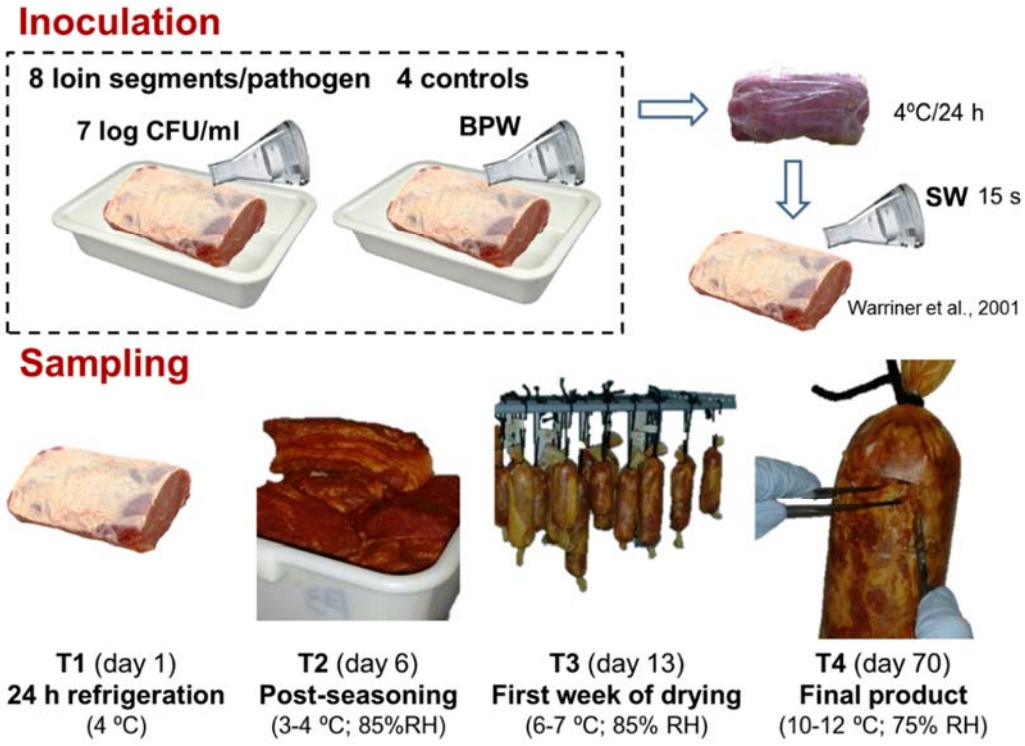

Fig. 2. Inoculation procedure, sampling and processing of the dry-cured pork loins along the study. BPW: sterile buffered peptone water. SW: sterile water. RH: relative humidity.

Acknowledgements

This study was financially supported by the Innovation and Development Agency the Andalusian Government (IDEA Agency; project references 351504/550859). Gómez-Laguna J is supported by a "Ramón y Cajal" contract of the Spanish Ministry of Economy and Competitiveness. Cardoso-Toset F is funded by a grant of the Agrifood Campus of International Excellence Programme (ceiA3) from the Ministry of Education, Culture and Sport and by the Santander Universities Global Division.

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Capítulo IV / Chapter IV:

Conclusiones / Conclusions

CONCLUSIÓN 1

Se demuestra la elevada prevalencia de *Salmonella* spp*., Campylobacter* spp*. y L. monocytogenes* en cerdos ibéricos criados en la dehesa, con la prevalencia más alta en amígdalas para *Salmonella* spp. (30,67%, IC95 23,85-38,44%) y *L. monocytogenes* (39,33%, CI95 31,87-47,32%) y en heces para *Campylobacter* spp. (57.33%, CI95 49.33-64.96%) (Objetivo primero; Estudio 1).

Se observó una alta diversidad solo en los aislamientos de *Salmonella* de muestras de cerdos en el matadero, con evidencia de contaminación cruzada a lo largo de la cadena de producción en el caso de este patógeno. (Objetivo primero; Estudio 1).

Las etapas previas al sacrificio (en la granja, transporte o la estabulación) y los procedimientos durante el sacrificio y faenado, incluyendo el sellado del recto antes de la evisceración intestinal y los protocolos de desinfección hidroalcohólica, se identificaron como estrategias de control críticas para reducir la contaminación de la carne de cerdo por *Salmonella* spp., *Campylobacter* spp. y *L. monocytogenes.* (Objetivo primero; Estudio 1).

CONCLUSIÓN 2

La alimentación con una dieta suplementada con un aditivo a base de *Pediococcus acidilactici* MA18 / 5M en la fase de finalización logró reducir la carga de *Campylobacter* spp.en las heces de cerdos ibéricos criados en extensivo. (Objetivo 2; Estudio 2).

CONCLUSIÓN 3

La concentración inicial de bacterias; la reducción progresiva del pH y la reducción de los valores de aw se consideran tres factores críticos en los cambios observados en *S. Typhimurium, C. coli* y *L. monocytogenes* a lo largo del proceso de curación de la caña de lomo de cerdo ibérico. (Objetivo 3; Estudio 3).

Aunque se observa una reducción de los recuentos bacterianos durante todo el proceso, los resultados de este estudio no respaldan que el proceso de producción de lomos de cerdo curados en seco elimine completamente *S. Typhimurium y L. monocytogenes* en caso de alta contaminación inicial. (Objetivo 3; Estudio 3).

CONCLUSION 1

Salmonella spp., *Campylobacter* spp. and *L. monocytogenes* were recovered at different stages of the production chain, with the highest prevalence detected in tonsils for *Salmonella* spp. (30.67%, CI_{95} 23.85-38.44%) and *L. monocytogenes* (39.33%, CI_{95} 31.87-47.32%) and in faeces for *Campylobacter* spp. (57.33%, CI_{95} 49.33-64.96%).

A high diversity was observed only in *Salmonella* isolates recovered from free-range pig samples at the slaughterhouse, with evidence of cross-contamination along the production chain in the case of this pathogen. These findings were not observed for *Campylobacter* spp. and *L. monocytogenes* isolates.

Pre-slaughter stages (on the farm, transport or lairage) and procedures during slaughtering and quartering, including the sealing of the rectum before gut evisceration and hydroalcoholic disinfection protocols, were identified as critical control strategies to reduce free-range pork carcass contamination by *Salmonella* spp., *Campylobacter* spp., and *L. monocytogenes*.

CONCLUSION 2

Free-range pigs fed with a diet supplemented with a commercial additive containing *Pediococcus acidilactici* MA18/5M succeed in reducing the *Campylobacter* spp. load in the faeces of treated animals.

CONCLUSION 3

Three critical factors justify the changes observed in S. Typhimurium, C. coli and L. monocytogenes along the curing process: (i) the initial concentration of the bacteria; (ii) the progressive reduction of pH; and (iii) the reduction of aw values.

Although a reduction of bacterial counts throughout the process is observed, the results of this study do not support that the production process of dry-cured pork loins completely removes *S.* Typhimurium and *Listeria monocytogenes* in case of high initial contamination.

Capítulo V / Chapter V:

Resumen / Summary

Esta tesis doctoral se presenta como compendio de publicaciones de los trabajos derivados del proyecto "*SAFEPORK Zoonosis Alimentarias en el cerdo Ibérico: prevalencia y control para la obtención de alimentos seguros"*, financiado con el Fondo Europeo de Desarrollo Regional, y gestionado por la Agencia de Innovación y Desarrollo de Andalucía (IDEA) (referencia #351504).

El cerdo Ibérico finaliza su periodo de engorde principalmente en la dehesa, ecosistema que aporta grandes mejoras en lo que se refiere al bienestar de los animales, pero que por otro lado favorece la interacción con otras especies animales, tanto domésticas como silvestres, lo que hace que las medidas de bioseguridad sean de más difícil adopción en las explotaciones de cerdo Ibérico, aumentando la susceptibilidad frente a determinados patógenos zoonósicos.

Entre los principales agentes zoonósicos alimentarios descritos por la EFSA destacan *Campylobacter* spp., *Salmonella* spp. y *Listeria monocytogenes*.

La importancia de estos zoonósicos alimentarios tanto desde el punto de vista de la salud pública como desde el punto de vista comercial, a la hora de abrir fronteras comerciales, hace necesario la consideración de este problema de manera global en las distintas áreas de producción del cerdo Ibérico con el fin de determinar el flujo de dichos agentes e implantar medidas para reducir su incidencia y por tanto garantizar la obtención de productos que no pongan en riesgo la salud del consumidor.

El primer objetivo de esta tesis doctoral ha sido determinar la prevalencia de los distintos patógenos zoonósicos que afectan al cerdo Ibérico en la dehesa mediante estudio de los animales sacrificados en matadero. Para ello se ha desarrollado el trabajo (Morales-Partera et al., 2018, *Food Control*, 92, 208-215) que evaluó la prevalencia y diversidad de *Salmonella* spp., *Campylobacter* spp. y *Listeria monocytogenes* a lo largo de la cadena de producción del cerdo Ibérico en matadero. Para ello se recolectaron un total

de 750 muestras (5 muestras / animal; 15 animales / granja; 5 granjas / matadero) de dos mataderos y se analizaron mediante metodologías ISO específicas los distintos patógenos. *Salmonella* spp. (12,93%; IC_{95} 10,72-15.52%), *Campylobacter* spp. (17,17%, IC_{95} 13,00-21,74%) y *L. monocytogenes* (9,07%; IC_{95} 7,21-11,33%) fueron identificados en diferentes fases de la cadena de producción, con la prevalencia más alta detectada en las tonsilas para *Salmonella spp*. (30,67%; IC_{95} 23,85-38,44%) y *L. monocytogenes* (39,33%, IC_{95} 31,87-47,32%) y en heces para *Campylobacter* spp. (57,33%; IC_{95} 49,33-64,96%). Se detectaron trece serotipos diferentes de *Salmonella*, siendo la *Salmonella* Typhimurium monofásica la más frecuente. *C. coli*, *C. jejuni* y *L. monocytogenes*, serotipos 4b y 1 / 2a, también fueron identificados.

En cuanto a la prevalencia total de *Salmonella* spp. en muestras de piel fue significativamente mayor en el matadero B que en el A. Además, se observó una prevalencia más alta, aunque no significativa, de los patógenos seleccionados en muestras de carne del matadero B con respecto al matadero A (10,67% vs 0% para *Campylobacter* spp.; y 4% vs 0% para *Salmonella* spp. y *L. monocytogenes*). Nuestros resultados resaltan el riesgo de contaminación de la carne de cerdo por los microorganismos en estudio y señalan la importancia de implementar medidas de control específicas.

El segundo objetivo ha consistido en aplicar medidas correctoras que permitan disminuir la prevalencia de los distintos agentes zoonósicos alimentarios de interés. Para esto se ha llevado a cabo el trabajo (Morales-Partera et al., 2019, actualmente en revisión). Este estudio evaluó el efecto de *Pediococcus acidilactici* como alternativa a los antibióticos y su impacto en los parámetros productivos y la microbiota fecal de cerdos en fase de engorde. Un total de 57 cerdos se seleccionaron al azar y se distribuyeron por peso en dos grupos: (1) grupo control, con un peso corporal inicial de 143,28 ± 14,26 kg que recibió una dieta control; y (2) grupo tratado, con un peso corporal inicial de 146,41 ± 13,71 kg que recibió la dieta de control suplementada con *Pediococcus acidilactici* durante los últimos 31 días de la etapa final antes de su llegada a matadero. En los días 0, 22 y 31 desde el inicio del estudio, se registraron el peso y la ganancia media diaria (ADG). A los 0 y 31 días se recogieron muestras fecales individuales de 8 cerdos por grupo para el recuento de enterobacterias, *Escherichia coli*, *Campylobacter* spp., bacterias del ácido láctico y detección de *Salmonella* spp. Se observó una reducción significativa en el recuento de *Campylobacter* spp. en animales tratados (P = 0.004) en

comparación con el grupo de control. Este estudio indica que la suplementación con *Pediococcus acidilactici* representa una aproximación útil para controlar la carga de *Campylobacter* spp.en el tracto intestinal en cerdos criados en extensivo antes de su llegada a matadero.

El tercer y último objetivo de esta tesis doctoral ha sido determinar la viabilidad de los patógenos seleccionados frente a las condiciones de curación empleadas en los productos del cerdo Ibérico. Para ello se llevó a cabo el trabajo (Morales-Partera et al., 2017, *Int J Food Microbiol*, 258, 68-72), en el que se analiza la eficacia del proceso de curación de los lomos de cerdos Ibéricos criados en extensivo en la reducción de tres de los patógenos más importantes transmitidos por los alimentos. Un total de 28 segmentos de lomo de cerdo, con un peso promedio de 0,57 ± 0,12 kg, se dividieron en cuatro grupos, tres de ellos inoculados por inmersión con 7 log CFU / ml de *Salmonella* Typhimurium, *Campylobacter coli* o *Listeria innocua* y el último se inoculó por inmersión con medio estéril (grupo control). Los lomos se trataron con una mezcla de aditivos y especias propios del curado, se embutieron y se curaron durante 64 días. Se evaluó el análisis microbiológico, el pH y la actividad del agua (a_w) en cuatro etapas. Como se esperaba, los valores de pH y a_w disminuyeron con el tiempo de curado. *S.* Typhimurium y *C. coli* disminuyeron significativamente (3,28 y 2,14 unidades logarítmicas, respectivamente), pero se observó una reducción limitada de *L. innocua* (0,84 unidad logarítmica) a lo largo del proceso de curado. En nuestro estudio, tres factores se consideraron críticos para la viabilidad de los patógenos estudiados a lo largo del proceso de curación: (i) la concentración inicial de las bacterias, (ii) la reducción progresiva del pH y (iii) la reducción de los valores de a_w. Nuestros resultados animan al incremento de la presión de muestreo en diferentes etapas del proceso de producción de la caña de lomo para garantizar la ausencia de los tres patógenos.

This PhD is presented as a compendium of publications from the research project "*SAFEPORK Food Zoonoses in the Iberian pig: prevalence and control to obtain safe food"*, financed with the European Regional Development Fund, and managed by the Agency for Innovation and Development of Andalusia (IDEA) (reference #351504).

The Iberian pig finishes its fattening period mainly in the Dehesa, an ecosystem that provides great improvements in terms of animal welfare, but on the other hand favors the interaction with other animal species, both domestic and wild, which makes that biosecurity measures are more difficult to be adopted in Iberian pig farms, increasing susceptibility to certain zoonotic pathogens.

Among the main food zoonotic agents described by EFSA we can find *Campylobacter* spp., *Salmonella* spp. and *Listeria monocytogenes*.

The importance of these food-borne zoonotic pathogens both from the public health and from the commercial points of view, when opening commercial borders, makes it necessary to consider this problem globally in the different areas of production of the Iberian pig in order to determine the circulation of these agents and implement measures to reduce their incidence and therefore ensure the procurement of products that do not endanger the health of the consumer.

The first aim of this PhD thesis was to determine the prevalence of the different zoonotic pathogens that affect the Iberian pig in the dehesa by studying slaughtered animals (Morales-Partera et al., 2018, *Food Control*, 92, 208-215). To evaluate the prevalence and diversity of these microorganisms along the free-range pork production chain, a total of 750 samples (5 samples/animal; 15 animals/farm; 5 farms/slaughterhouse) were collected from two slaughterhouses and analysed by specific ISO methodologies. *Salmonella* spp*.* (12.93%, CI_{95} 10.72-15.52%), *Campylobacter* spp.

(17.17%, CI_{95} 13.00-21.74%) and *L. monocytogenes* (9.07%, CI_{95} 7.21-11.33%) were recovered at different stages of the production chain, with the highest prevalence detected in tonsils for *Salmonella* spp. (30.67%, CI_{95} 23.85-38.44%) and *L. monocytogenes* (39.33%, CI_{95} 31.87-47.32%) and in faeces for *Campylobacter* spp. (57.33%, CI_{95} 49.33-64.96%). Thirteen different *Salmonella* serotypes were detected with monophasic *Salmonella* Typhimurium as the most frequent one. *C. coli*, *C. jejuni* and *L. monocytogenes* serotype 4b and 1/2a were also identified. A significant higher prevalence of *Salmonella* spp. in total and from skin samples in slaughterhouse B than in slaughterhouse A was detected. In addition, a higher, although not significant, prevalence of the selected pathogens was observed in meat samples from slaughterhouse B with respect to slaughterhouse A (10.67% vs 0% for *Campylobacter* spp.; and 4% vs 0% for *Salmonella* spp. and *L. monocytogenes*). Our results highlight the risk of contamination of pork meat by the microorganisms under study and point out the importance of implementing specific control measures.

The second aim of this PhD thesis was to apply corrective measures to reduce the prevalence of zoonotic agents detected along the production chain (Morales-Partera et al., 2019, *under review*). This study evaluated the effect of *Pediococcus acidilactici* as alternative to antibiotics and its impact on performance parameters and faecal microbiota of finishing pigs. A total of 57 free-range pigs were randomly allocated and blocked by weight into two groups: (1) control group, with an initial BW of 143.28±14.26 kg which received a control diet; and (2) treated group, with an initial BW of 146.41±13.71 kg which received the control diet supplemented with *Pediococcus acidilactici* during the last 31 days of the finishing stage prior to their delivery to the slaughterhouse. At days 0, 22 and 31 from the beginning of the study weight and average daily gain (ADG) were recorded. Individual faecal samples from 8 pigs per group at 0 and 31 days were analysed for enumeration of *Enterobacteria, Escherichia coli, Campylobacter* spp., lactic acid bacteria and detection of *Salmonella* spp. A significant decrease in the counts of *Campylobacter* spp. in treated animals (P=0.004) in comparison with the control group was detected. This study indicates that supplementation with *Pediococcus acidilactici* may represent a useful approach to control *Campylobacter* spp. load in the intestinal tract of free-range finishing pigs prior to slaughter.

The third objective of this PhD thesis was to determine the viability of selected

pathogens against dry-curing conditions used in Iberian pig products (Morales-Partera et al., 2017, *Int J Food Microbiol*, 258, 68-72). In this study, the efficacy of the dry curing process of pork loins obtained from free-range pigs in the reduction of three of the most important foodborne pathogens is analysed. A total of 28 pork loin segments, with an average weight of 0.57 ± 0.12 kg, were divided into four groups with three being inoculated by immersion with 7 log CFU/ml of either *Salmonella* Typhimurium*, Campylobacter coli* or *Listeria innocua* and the last one inoculated by immersion with sterile medium (control group). The loin segments were treated with a seasoning mixture of curing agents and spices, packed in a synthetic sausage casing and cured for 64 days. Microbiological analysis, pH and water activity (a_w) were assessed at four stages. The values of pH and a_w decreased with curing time as expected. *S.* Typhimurium and *C. coli* dropped significantly (3.28 and 2.14 log units, respectively), but limited reduction of *L. innocua* (0.84 log unit) was observed along the curing process. In our study, three factors were considered critical: the initial concentration of the bacteria, the progressive reduction of pH and the reduction of a_w values. Our results encourage performing periodic analysis at different stages of the manufacturing of dry-cured pork loins to ensure the absence of the three evaluated foodborne pathogens

Indicios de calidad

INDICIOS DE CALIDAD DE LA TESIS APORTADOS POR EL DOCTORANDO

APELLIDOS	NOMBRE
MORALES PARTERA	ANGELA

TÍTULO DE LA TESIS

ZOONOSIS ALIMENTARIAS EN EL CERDO IBÉRICO: PREVALENCIA Y CONTROL EN LA GRANJA PARA LA OBTENCIÓN DE ALIMENTOS SEGUROS.

☐ Especificar la publicación que aporta como indicios de calidad de la tesis, establecidos en el artº. 25.a de las actuales normas reguladoras de los estudios de doctorado:

- Título: ***Prevelence and diversity of Salmonella spp., campylobacter spp. and Listeria monocytogenes in two free-range pig slaughterhouses.***

- Autores (p.o. de firma): *A.M. Morales-Partera, F. Cardoso-Toset, I. Luque, R.J. Astorga, A.Maldonado, S. Herrera León, M.Hernández, J. Gómez-Laguna, C. Tarradas.*

- Revista (año,vol.,pág.): *Food Control* (2018) 208-2015

- Base de Datos Internacional o Nacional) en las que está indexada: JCR

- Área temática en la Base de Datos de referencia: Food Science and Technology

- Índice de impacto de la revista en el año de publicación del Artículo: 4,248

- Lugar que ocupa/Nº de revistas del Área temática: 11/135

- Título: ***Survival of selected foodborne pathogens on dry cured porks loins.***

- Autores (p.o. de firma): *A.M. Morales-Partera, F. Cardoso-Toset, F. Jurado-Martos, R.J. Astorga, B. Huerta, I. Luque, C. Tarrada, J. Gómez-Laguna.*

- Revista (año,vol.,pág.): *International Journal of Food Microbiology (2017) 258 68-72*

- Base de Datos Internacional o Nacional en las que está indexada: JCR

- Área temática en la Base de Datos de referencia: Food Science and Technology

- Índice de impacto de la revista en el año de publicación del Artículo: 3,451

- Lugar que ocupa/Nº de revistas del Área temática: 17/133

☐ Internacionalización tesis : presenta la tesis con mención internacional y aporta contribuciones científicas no incluidas como publicaciones

SR/A. COORDINADOR/A DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORA

8th March 2016

To whom it may concern,

This letter is to confirm that **Ms. Angela Maria Morales Partera**, enrolled on the Agrifood Biosciences and Sciences Doctoral Program (regulated by RD 1393/2007), at University of Cordoba, Spain, has worked from **September 10, 2013 to December 19, 2013** in the Built Environment and Sustainable Technologies (BEST) Research Institute, Faculty of Technology and Environment, Liverpool John Moores University, Liverpool, UK.

While there she demonstrated that she has obtained the necessary practical and theoretical requirements to perform microwave rotational spectroscopy analysis of food products. This included learning and familiarization of different microwave frequency equipment (e.g. vector network analyser, oscilloscope, and spectrum analyser), implementing numerous different analysis methodologies to study the dielectric characteristics of samples (e.g. resonant/non-resonant, reflection/transmission and contact/non-contact) and collating, interpreting and relating the resulting raw data to find the association with meat characteristics. The developed sensors and methodologies

based upon microwave technology were applied to the control of food safety in meat products. Namely, the quantification of water activity (aw), the detection of pathogenic microorganisms and assessing the spoilage of Iberican pig products.

Development of the sensor involved using current physicochemical and microbiological methodologies in a_w prediction, pathogenic microorganism differentiation and quantification, and spoilage parameters using numerous experimental designs to establish correlations between the raw data of the microwave methodologies and the outcome of current methodologies (i.e. Aqualab, ISO microbiology methods and TBARS and sensory test).

In her time at the BEST Research Institute she showed to be productive and able despite the differences between her native scientific field of microbiology and the engineering/theoretical physics techniques required to implement the above methodologies, demonstrating academic flexibility and her adaptive qualities.

It should also be noted that Ms. Morales was an enthusiastic, open-minded academic who fully engaged with the undertaken research while being friendly and working well with the research staff and is very promising as a research worker in the field of veterinary microbiology.

Yours Sincerely,

Dr Alex Mason *BSc, PhD, PGCE, FHEA, MIET, CEng*

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Otras aportaciones científicas /
Other contributions

La Tesis Doctoral que presentamos es un ejemplo claro de investigación colaborativa, entendiendo por investigación colaborativa aquella realizada por varias entidades, generalmente de diferente naturaleza, que comparten el interés por la ejecución de un proyecto y el esfuerzo por desarrollarlo, también comparten los riesgos y la propiedad de los resultados conforme a protocolos de titularidad previamente acordados (Casals et al., 2008).

Además de los trabajos indexados resultados de la Tesis y que están descritos en los objetivos anteriormente expuestos, es importante resaltar que en el proceso de Transferencia del Conocimiento uno de los elementos fundamentales es la difusión de los resultados al sector productivo y a la sociedad en general.

Esta Tesis es fruto de una colaboración estrecha entre la Universidad un Centro Tecnológico Agroalimentario (CICAP) y la empresa, de hecho uno de los codirectores es Investigador del Departamento de I+D+i del CICAP. La participación y colaboración de varias instituciones enriquece sin duda el desarrollo de este trabajo, además de garantizar la publicación, difusión y utilización de los resultados obtenidos. Una de las principales herramientas en la difusión de los resultados es la participación en foros, congresos, reuniones y symposium específicos, ampliamente desarrollada en esta Tesis Doctoral y cuyas principales aportaciones se muestran a continuación:

Microwave dielectric spectroscopy – A versatile methodology for online, non-destructive food analysis, monitoring and process control. R.T. Blakey, A.M. Morales-Partera. *Engineering in Agriculture, Environment and Food*, 2016. 9(3): p. 264-273.

ARTICLE IN PRESS

Engineering in Agriculture, Environment and Food xxx (2016) 1–10

Contents lists available at ScienceDirect

Engineering in Agriculture, Environment and Food

journal homepage: http://www.sciencedirect.com/eaef

Microwave dielectric spectroscopy – A versatile methodology for online, non-destructive food analysis, monitoring and process control

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ARTICLE INFO

Article history:
Received 8 June 2015
Received in revised form
12 October 2015
Accepted 3 February 2016
Available online xxx

Keywords:
Microwave
Dielectric
Spectroscopy
Food
Analysis
Sensors
Quality
Safety

ABSTRACT

Microwave dielectric spectroscopy (MDS) is an online, compact, non-destructive/invasive, low power analytical methodology based upon the rotation of molecules and their functional groups in the presence of an electromagnetic field in the frequency range of 0.3–300 GHz which may then be used to differentiate materials of different composition. Recent technological developments have increased the availability of the equipment needed to investigate the application of MDS within the food industry. This article gives an overview of the fundamentals of the technology and a review of potential applications in the food industry. Challenges and the future potential of this technology are also considered.

Contents

1. Introduction

The microwave region of the electromagnetic spectrum extends from 300 MHz to 300 GHz and is more widely known for communication, radar and heating applications. However, the application of the fundamental concept of these technologies can

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http://dx.doi.org/10.1016/j.eaef.2016.02.001

Please cite this article in press as: Blakey, R.T., Morales-Partera, A.M., Microwave dielectric spectroscopy – A versatile methodology for online, non-destructive food analysis, monitoring and process control, Engineering in Agriculture, Environment and Food (2016), http://dx.doi.org/10.1016/j.eaef.2016.02.001

Interannual variability on seroprevalence of *Salmonella* spp. in free-range fattening pigs in South Spain. Reguillo L., *Morales A.,* Astorga R.J., Hernández M., Luque I., Tarradas C., Gómez-Laguna J.*5th European Symposium of Porcine Health Management ESPHM*. Edimburgo, 2013.

Interannual variability on seroprevalence of *Salmonella* spp. in free-range fattening pigs in South Spain

Reguillo L [1], Morales-Partera A [2], Astorga R J [1], Hernández M [2], Luque I [1], Tarradas C [1], Gómez-Laguna J [2]

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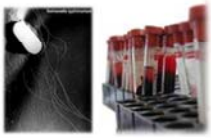
OBJECTIVE

The aim of this study was to evaluate the effect of interannual variability on *Salmonella* seroprevalence at herd and individual levels in pigs reared in free-range systems in South Spain.



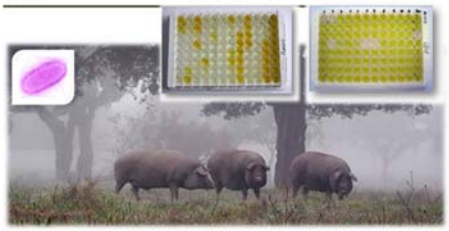
MATERIAL & METHODS

To carry out this study blood samples from 10-15 animals/herd, from 62 and 40 herds, which belonged to the same stockbreeding cooperative, were collected at the abbatoir. The interannual variability was calculated from samples collected during the season January-April from 2008 (62 herds) and 2011 (40 herds). Serum samples were analysed to detect specific antibodies against *Salmonella* spp. using the same commercial ELISA kit (SalmotypePig Screen+E, Labor Diagnostik Leipzig, Leipzig, Germany; cut-off >40%).

RESULTS

Table 1. Number of positive swine sera against *anti-Salmonella* antibodies by means of ELISA in 2008 and 2011

	2008	2011
Positive pigs	136/620	350/599
Individual Prevalence	21.93%	58.43%
CI_{95}	18.69 -25.19	54.49-62.37
Positive herds	48/62	40/40
Herd Prevalence	77.42%	100%
CI_{95}	67.02-87.72	-



CONCLUSION

The marked increased observed in the seroprevalence of *Salmonella* in our study highlights the importance of including interannual surveys in free-range pigs, pointing out to the analysis of serum samples as a useful tool for surveillance and control studies on *Salmonella*.

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Serosurvey of *Salmonella* spp. and *Yersinia* spp. in fattening pigs reared in free-range systems. Morales A., J. Herrera, F. Cardoso-Toset, L. Gómez-Gascón, C. Tarradas, B. Huerta, J. Gómez-Laguna. *5th European Symposium of Porcine Health Management ESPHM*. Edimburgo, 2013

22nd - 24th ay 2013 Edinburgh, UK

Serosurvey of *Salmonella* spp. and *Yersinia* spp. in fattening pigs reared in free-range systems

Morales A.[1], Herrera J.[1], Cardoso-Toset F.[2], Gómez Gascón L.[2], Luque I.[2], Huerta B.[2], Gómez-Laguna J. [1]

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INTRODUCTION & OBJECTIVE

Zoonotic agents, such as *Salmonella* spp. and *Yersinia* spp., are considered high-risk zoonotic pathogens by the European Food Safety Agency (EFSA) and have a significant public health impact. Determining the prevalence of these pathogens represents a useful tool for establishing basic control measures directed to reduce their impact. Thus, the aim of this study was to evaluate the seroprevalence of *Salmonella* spp. and *Yersinia* spp. in pigs reared in outdoor systems from South Spain.

MATERIAL & METHODS

A total of 1187 serum samples were collected at two different abattoirs. Approximately, 15 animals/herd were samples belonging to 80 different herds (40 herds/abbatoir). Serum samples were analysed for specific antibodies against *Salmonella* spp. and *Yersinia* spp. using commercial ELISA kits (SalmotypePig Screen+E, and, PigType YopScreen, Labor Diagnostik Leipzig, Leipzig, Germany)

RESULTS

Table 1: Individual prevalence, herd prevalence and intraherd prevalence for *Salmonella* spp and *Yersinia* spp:

	Yersinia spp.				*Salmonella* spp.			
	A		B		A		B	
	Cut off (%) 20%	Cut off (%) 40%	Cut off (%) 20%	Cut off (%) 40%	Cut off (%) 20%	Cut off (%) 40%	Cut off (%) 20%	Cut off (%) 40%
INDIVIDUAL PREVALENCE%	94,16	82,14	93,03	81,29	87,81	58,43	89,46	54,25
HERD PREVALENCE %	100,00	100,00	100,00	100,00	100,00	100,00	100,00	100,00
INTRAHERD PREVALENCE %	(46,67-100)	(13,33-100)	(26,67-100)	(13,33-100)	(40-100)	(6,67-100)	(41,67-100)	(13,33-100)
median (%)	100,00	86,67	100,00	83,33	93,33	60,00	93,33	53,33

CONCLUSION

A high risk of infection posed by *Salmonella* spp. and/or *Yersinia* spp. might be expected from the targeted population of this study due to their high seroprevalence, however, it is not feasible to guarantee an association between the high seroprevalence observed with the presence of these organisms in meat products

AKNOWLEDGEMENTS: This study was financially supported by the Department of Innovation, Science and Enterprise of the devolved regional government of Andalusia (Agencia IDEA) of Spain, project number 351504. FCT is funded by the International Campus of Excellence Programme, from the Ministry of Education, Culture and Sport and by Santander Universities Global Division.

Prevalence of *Campylobacter* spp., *Salmonella* spp., and *Listeria monocytogenes* in two free range pig slaughterhouses. *Morales A.,* Cardoso-Toset F., Luque I., Fernández L., Tarradas C., Astorga R.J., Hernández M., Gómez-Laguna J. *6th European Symposium of Porcine Health and Management*, Sorrento, Italy, 2014

7th - 9th May 2014 Sorrento, Italy

Prevalence of *Campylobacter* spp., *Salmonella* spp. and *Listeria monocytogenes* in two free-range pig slaughterhouses

Morales A.[1], Cardoso-Toset F.[1,2], Luque I.[2], Fernández L.[1], Tarradas C.[2], Astorga R.J.[2], Hernández M.[1], Gómez-Laguna J.[1]

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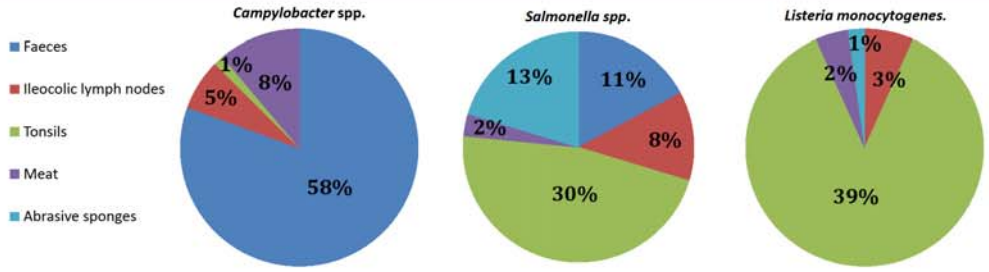
INTRODUCTION & OBJECTIVE

Zoonotic agents, such as *Campylobacter* spp., *Salmonella* spp. and *Listeria monocytogenes* are considered high-risk zoonotic pathogens by the European Food Safety Agency (EFSA) and have a significant impact on public health. The aim of this study was to determine the prevalence of these pathogens along the free-range pig production chain of two slaughterhouses from Spain.

MATERIAL & METHODS

Five farms from each slaughterhouse were selected based on their high *Salmonella* spp. seroprevalence in previous studies. A total of 150 animals (fifteen animals/farm) were traced to collect a total of five samples/animal (faeces, abrasive sponges at the pre-scalding point, ileocolic lymph nodes, tonsils and meat samples; a pool from ham, loin and shoulder). All samples were analysed by means using specific ISO methodologies (UNE-EN:ISO 6579:2002; 10272-1:2006; 11290-2:2000) for the detection of the above mentioned pathogens.

RESULTS



Campylobacter spp. was the most prevalent agent in faeces, followed by *Salmonella* spp.; whereas *Listeria monocytogenes* was not detected. Contrary, *Listeria monocytogenes* was the most prevalent agent in samples collected from the tonsils, followed by *Salmonella* spp., which suggests a high circulation of these pathogens at pre-slaughter level and point to a potential environmental cross-contamination. The prevalence of the examined agents was similar in both slaughterhouses, but for meat samples (0% *vs* 16% for *Campylobacter* spp.; 0% *vs* 4% for *Salmonella* spp. and *Listeria monocytogenes*), that were only observed in one slaughterhouse, where some controls measures, such as sealing off the rectum is not performed.

CONCLUSION

Our results highlight a low risk of infection posed by *Campylobacter* spp., *Salmonella* spp. and *Listeria monocytogenes* in meat from free-range pigs, and point out the necessity of making compulsory strict control measures along the slaughterline in order to assure a lower pork contamination by these pathogens.

AKNOWLEDGEMENTS: This study was financially supported by the Department of Innovation, Science and Enterprise of the devolved regional government of Andalusia (Agencia IDEA) of Spain, project number 351504. FCT is funded by the International Campus of Excellence Programme, from the Ministry of Education, Culture and Sport and by Santander Universities Global Division.

Efficacy hydroalcoholic disinfection on *Salmonella* prevalence in a pig slaughterhouse. Morales A., Cardoso-Toset F., Hernández M., Luque I., Fernández L., Gómez-Laguna J., Astorga R.J. *6th European Symposium of Porcine Health and Management*, Sorrento, Italy, 2014

7th - 9th May 2014 Sorrento, Italy

Efficacy of hydroalcoholic disinfection on Salmonella prevalence in a pig slaughterhouse

Morales A.[1], Cardoso-Toset F.[1,2], Hernández M.[1], Luque I.[2], Fernández L.[1], Gómez-Laguna J.[1], Astorga R.J.[2]

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INTRODUCTION & OBJECTIVE

***Salmonella* spp. is considered one of the most important zoonotic pathogens by the European Food Safety Agency (EFSA). In foods, it is frequently found in eggs and raw meat from pigs, turkeys and chickens. The surveillance of *Salmonella* in animals and foods is essential for advising on possible control and prevalence reduction measurements. This communication summarises the main results of two studies to evaluate the effect of hydroalcoholic disinfection on *Salmonella* prevalence and serotypes isolated from animals in a free range pig slaughterhouse from Spain.**

MATERIAL & METHODS

The first study was carried out in 2010 and a total of 8 farms were sampled (10 animals/farm). In the second study 5 farms were sampled during 2013 (15 animals/farm). In this second study a hydroalcoholic disinfection protocol was applied in spray on cutting surfaces at quartering at approximate intervals of 4 hours. Samples were analysed using the specific ISO methodology ISO 6579:2002 for the detection of *Salmonella* spp. and furthermore serotyped by means of agglutination techniques using commercially available antisera (Biorad). The samples were collected during the same season in 2010 and 2013.

RESULTS

Our results show a marked decrease in the global prevalence of *Salmonella* from 19.00% to 10.40% between 2010 and 2013, but for the tonsils samples which presented a relatively higher prevalence. Furthermore, different serotypes were identified in most of the sampled stages.

	Faeces	Pre-scalding	Ileocolic LN	Tonsil	Meat
2010	21.25%	36.25%	16.25%	17.50%	3.75%
	S. Derby, *S.* Rissen	*S.* Bredeney, *S.* Typhimurium	*S.* Derby, *S.* Rissen	*S.* Rissen, *S.* Israel, *S.* Derby	*S.* Rissen, *S.* Bredeney
2013	14.67%	2.67%	5.33%	29.33%	0%
	S. Typhimurium, *S.* Hessarek	*S.* Typhimurium (m)	*S.* Typhimurium (m)	*S.* Hessarek, *S.* Typhimurium (m)	-

CONCLUSION

This finding, together with the still high prevalence of *Salmonella* spp. in faeces suggest a high circulation of *Salmonella* and indicate a potential environmental cross-contamination. Nonetheless, the hydroalcoholic disinfection protocol implemented in our study was successful in eliminating *Salmonella* spp. circulation at quartering. Furthermore, a shift in *Salmonella* serotypes was detected to a higher prevalence of *S.* Hessarek, *S.* Typhimurium and monophasic *S.* Thyphimurium (mST). Although inter-annual variability must be taken into account, our results highlight that disinfection protocols with hydroalcoholic products may represent a useful control measure to minimize *Salmonella* circulation from pork.

AKNOWLEDGEMENTS: This study was financially supported by the Department of Innovation, Science and Enterprise of the devolved regional government of Andalusia (Agencia IDEA) of Spain, project number 351504. FCT is funded by the International Campus of Excellence Programme, from the Ministry of Education, Culture and Sport and by Santander Universities Global Division.

Comportamiento de *Salmonella* spp., *Listeria* spp. y *Campylobacter coli* durante el proceso productivo de la caña de lomo ibérica. Morales-Partera, A.; F. Cardoso-Toset, F. Jurado-Martos, A. Galán-Relaño, B. Barrero, A. Maldonado, I. Luque, C.Tarradas, J. Gómez-Laguna. *III Congreso Internacional de Seguridad Alimentaria*. Murcia, 2015.

Comportamiento de *Salmonella*, spp., *Listeria spp. y Campylobacter coli* durante el proceso productivo de la caña de lomo ibérica.

Morales-Partera , A. [1], Cardoso-Toset, F.[1,2], Jurado-Martos, F.[1], Galan-Relaño, A. [1], Barrero, B. [1], Maldonado, A. [1],Luque,I. [1], Tarradas, C. [1], Gómez-Laguna, J. [2]

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Introducción y Objetivos

La carne de cerdo y derivados, especialmente los productos curados, como la caña de lomo, presentan actualmente una gran demanda por los consumidores. Los embutidos son productos elaborados mediante un proceso tecnológico que incluye la adición de aditivos (fundamentalmente sal, sales nitrificantes y adobo) y una posterior maduración en cámaras sometidas a condiciones de temperatura y humedad determinadas, que parecen inactivar los microorganismos patógenos. Dada la gran variabilidad de productos curados, es necesario conocer como afecta este proceso a la viabilidad de los principales agentes implicados en las Enfermedades de Transmisión Alimentaria (ETAS) (Lindqvist and Lindblad,2003; Stollewerk et al., 2012, EFSA, 2015). .

El objetivo de este trabajo es estudiar el comportamiento de *Salmonella spp.* , *Listeria spp.* y *Campylobacter coli* durante el proceso productivo de la caña de lomo ibérica.

Material y Métodos

Se utilizaron 56 piezas de lomo, 16 por cada microorganismo y 8 controles negativos no inoculados. La inoculación de las piezas se realizó por inmersión en una solución que contenía caldo Brain Heart Infusion con el patógeno en estudio. Tras la maceración, todas las piezas fueron embutidas con una envoltura sintética de colágeno y se mantuvieron a 6-7ºC y 85% de Humedad Relativa (HR) durante una semana y a 11 ºC y 75% HR durante 50 días. Se establecieron cuatro tiempos de estudio (post-inoculación T1, post-maceración T2, primera semana de curación T3 y producto final T4) , tomando muestras de la zona más superficial. Se realizaron dos tipos de análisis microbiológico, recuento bacteriano y presencia/ausencia, siguiendo la metodología UNE-ISO correspondiente a cada bacteria (UNE-EN ISO 6579:2002, 11290-2:2000 and 10272-1:2006) así como medidas de pH y actividad de agua (a_w) para cada una de las muestras

Resultados y Discusión

Se observó una reducción de *Salmonella spp.* en todas las fases analizadas (T1-T4), como se aprecia en la Figura 1, obteniéndose el mayor porcentaje de reducción de este patógeno en la última fase (62,68%). El recuento inicial de *Listeria spp.* fue de 6,21±0,88 log ufc/g, obteniéndose una reducción limitada en la fase final. Los recuentos bacterianos obtenidos en T2 y T3 mostraron un comportamiento fluctuante para este microorganismo, obteniéndose el mayor porcentaje de reducción en T2, con un incremento de esta bacteria en T3 y reducción del contaje en T4 (Figura 1).

Los recuentos iniciales de *Campylobacter coli* fueron más bajos que otros patógenos analizados (2,14±0,19 log ufc/g) y se observó una reducción importante en todo el proceso. En la fase final, este microorganismo no pudo ser detectado en las muestras analizadas (Figura 1). Estudios previos demuestran la importancia de algunos factores protagonistas del proceso de curación en la inhibición de microorganismos, como temperatura, pH, aditivos y la actividad de agua de otros productos curados (Landeta et al., 2013).

Los valores de pH y actividad de agua (a_w) disminuyeron a lo largo del proceso de 5,85±0,18 a 5,56±0,12 y de 0,990±0,01 a 0,887±0,03 respectivamente , siendo los valores recomendados para este tipo de producto.

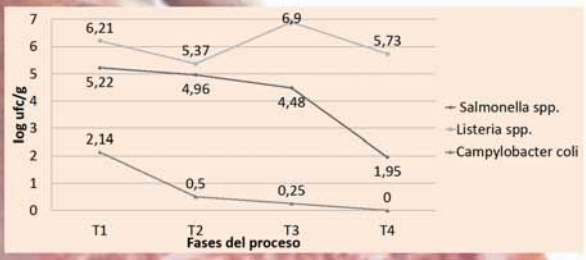

Figura 1. Evolución de log ufc/g en el proceso productivo de la caña de lomo ibérica de los microorganismos analizados.
***Nota:** Fases del proceso; post-inoculación T1, post-maceración T2, primera semana de curación T3 y producto final T4*

Conclusiones

A Nuestros resultados demuestran que el proceso productivo de la caña de lomo ibérica reduce la carga bacteriana de *Salmonella spp.*, *Listeria spp.* y *Campylobacter coli* bajo las condiciones estudiadas. En general, el efecto bactericida aumenta a medida que el proceso avanza por las diferentes fases.

B Se confirma la importancia de realizar un exhaustivo control de estos agentes en la producción primaria (granja), así como evitar las contaminaciones cruzadas en el matadero con la única finalidad de disminuir la prevalencia de estos microorganismos en la materia prima.

Autores

III Congreso Internacional de Seguridad Alimentaria **2015 Murcia**

Viabilidad de *Salmonella* spp, *Yersinia enterocolitica*, *Listeria* spp.y *Campylobacter coli* a diferentes concentraciones de sal y sales nitrificantes en un ensayo *in vitro*. , F. Jurado-Martos, A. Morales-Partera; B. Barrero, A. Galán-Relaño, L. Gómez Gascón, R.J. Astorga, B. Huerta, J.Gómez-Laguna C. Tarradas. *III Congreso Internacional de Seguridad Alimentaria*. Murcia, 2015.

Viabilidad de *Salmonella spp, Yersinia enterocolitica, Listeria spp.* y *Campylobacter coli* a diferentes concentraciones de sal y sales nitrificantes en un ensayo *in vitro*

Jurado-Martos, F.[1], Morales Partera, A.[1], Barrero, B[1], Galan-Relaño, A.[1], , Gomez-Gascón, L[1]., Astorga R.J[1]., Huerta, B.[1], Gómez-Laguna, J.[2], Tarradas, C.[1]
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Introducción y Objetivos

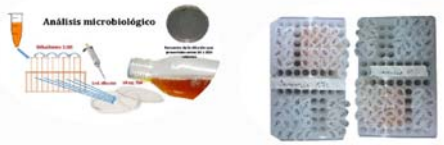
Los productos cárnicos curados se elaboran mediante un proceso tecnológico que incluye la adición de aditivos (fundamentalmente sal y sales nitrificantes) y una posterior maduración en cámaras sometidas a condiciones de temperatura y humedad determinadas. El uso tradicional de estas sales de curado se debe principalmente a su contribución en el sabor, olor y textura (Olense et al., 2004) y al papel que desempeñan como conservantes, evitando el deterioro que se produce por microorganismos, enzimas autolíticas, o la oxidación de lípidos entre otros (Sindelar y Milkowski, 2011; Mellefont et al., 2013).

Estas sales favorecen la reducción de la actividad agua y pH durante el proceso de curación (Parthasarathy y Bryan, 2012), factores que más influyen en la calidad microbiológica del producto, pero su efecto bactericida y/o bacteriostático es menos conocido (Tompkin, 2005).

El principal objetivo de este trabajo es realizar un estudio *in vitro* para determinar la viabilidad de *Salmonella* spp., *Yersinia spp.*, *Listeria* spp. y *Campylobacter coli*, como principales microorganismos implicados en los brotes de enfermedad causados por este tipo de productos, frente a diferentes concentraciones de sal y sales nitrificantes

Material y Métodos

Para realizar el ensayo se partió de una suspensión bacteriana en caldo *Brain Heart Infusion* para cada uno de los microorganismos a estudiar con una concentración aproximada de 10^7 UFC/mL. En eppendorf independientes se dispensaron 100 µL de inóculo con 900 µL de la concentración de aditivo; en el caso de la sal se estudiaron concentraciones comprendidas entre el 10 y el 0,5%, y para las sales nitrificantes entre el 20 y el 2,5%. Se establecieron 4 tiempos de estudio (0, 8, 24 horas y 1 semana), donde se realizó un recuento de unidades formadoras de colonias por mL (UFC/mL), sembrando diluciones seriadas en base 1/10 en Agar Tripticasa Soja (TSA). Se realizaron dos ensayos, para valorar el posible efecto de la temperatura, 10ºC y 37ºC.



Resultados y Discusión

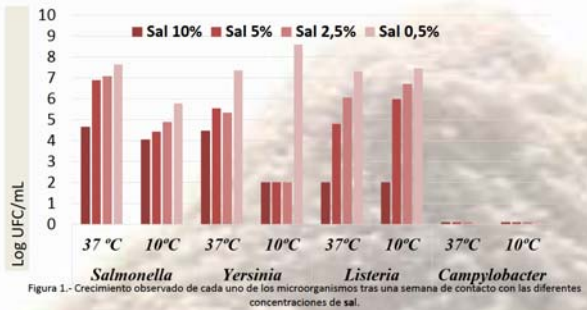


Figura 1.- Crecimiento observado de cada uno de los microorganismos tras una semana de contacto con las diferentes concentraciones de sal.

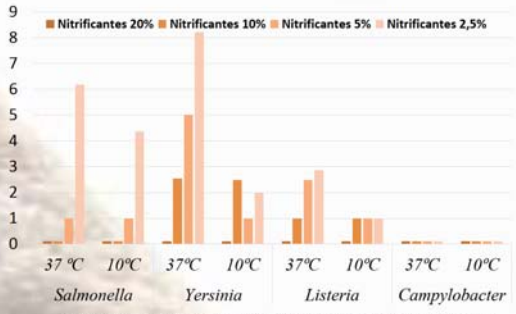


Figura 2.- Crecimiento observado de cada uno de los microorganismos tras una semana de contacto con las diferentes concentraciones de sales nitrificantes.

De forma general, se ha observado que los aditivos producen un aumento de la fase de latencia sobre la curva normal de crecimiento del microorganismo, y una disminución de los valores alcanzados en la fase de crecimiento exponencial. Como cabría esperar, las concentraciones más altas de sal (NaCl) y sales nitrificantes son las que presentan un mayor poder inhibidor del crecimiento. Las sales nitrificantes presentan un mayor efecto que la sal, pero no se observan diferencias significativas entre lo microorganismos analizados, aunque hay estudios que indican que las bacterias Gram positivas son más resistentes a la acción de la sal y de las sales nitrificantes que las bacterias Gram negativas (Mellefont et al., 2013).

En la Figura 1 se representan los recuentos obtenidos tras una semana de incubación. En el caso de la sal ninguna concentración fue capaz de producir un efecto bactericida total sobre los microorganismos estudiados (a excepción de *Campylobacter coli*). En otros estudios (Wijnker et al., 2006) se ha demostrado la supervivencia de *Salmonella* y *Listeria* durante 30 días a diferentes concentraciones de sal.

Las sales nitrificantes, (Figura 2) si presentaron un efecto bactericida total para una concentración de inóculo de 10^5 UFC/mL. Como indican algunos investigadores (Neubauer y Götz, 1996; Tompkin, 2005; Götz et al., 2006), el efecto de las sales nitrificantes está relacionado con una inhibición de las enzimas metabólicas, para ello requieren la presencia de otros compuestos como el hierro, con los que crea compuestos más tóxicos para los microorganismos. Esto sugiere que en un ensayo *in vivo* sobre productos cárnicos, el efecto bactericida de las sales nitrificantes tiene que ser mayor.

A En el estudio *in vitro* se comprueba que *Salmonella spp*, *Listeria spp.* y *Yersinia enterocolitica*, permanecen viables a concentraciones de sal (NaCl) y sales nitrificantes iguales o inferiores al 10%, a excepción de *Campylobacter coli*.

B La reducción de la carga bacteriana está relacionada con la concentración del aditivo, aunque también se asocia a otros factores, como la temperatura del ensayo (10 o 37 ºC). Estos resultados sugieren que el poder bactericida de los aditivos depende de un conjunto de factores que actuarían de forma sinérgica

III Congreso Internacional de Seguridad Alimentaria **2015 Murcia**

***Salmonella* prevalence and characterization in a free-range pig slaughterhouse in two periods (2010 and 2013).** Ángela Morales-Partera, Fernando Cardoso-Toset, Manuela Hernandez, Rafael J. Astorga, Inmaculada Luque, Carmen Tarradas, Silvia Hererra-León, Jaime Gómez-Laguna. *I3S International Symposium Salmonella and Salmonellosis*. Saint-Malo, France, 2016.

Salmonella PREVALENCE AND CHARACTERIZATION IN A FREE-RANGE PIG SLAUGHTERHOUSE IN TWO PERIODS (2010 and 2013)

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I. INTRODUCTION & OBJECTIVE

According to EFSA (2015) a downward trend of salmonellosis has been observed in Europe with products derived from chicken and pork meat being significant sources of this pathogen. Slaughterhouses provide many opportunities for the proliferation of pathogenic bacteria with contamination during bleeding, polishing, splitting, scalding and forced chilling. **This research summarises the changes observed in the inter-annual prevalence and serotypes of *Salmonella* and the effectiveness of rigorous cleaning procedures.**

II. MATERIAL & METHODS

A total of 375 samples were collected at the slaughterhouse at different stages of the production chain during the campaign of slaughter in 2013: i) post-stunning/pre-scalding, skin sample using abrasive sponges; ii) ileocecal lymph nodes, faeces and tonsils samples; iii) quartering, pooled meat samples from ham, loin and shoulder. Samples were obtained from animals belonging to 5 different herds (15 animals/farm) with a seroprevalence of >50% (positive cut off of 40%), considered as at high risk of exposure to *Salmonella* according to a previously established categorization (Mannion *et al.*, 2010).

During 2010 a systematic sampling from eight different free-range pig production units was carried out. Ten fattening pigs per herd were sampled. The traceability for each pig was strictly followed along the abattoir. Five different stages of the production chain and different samples were tested in each assay (Hernández *et al.*, 2013).

All samples were analysed by using specific ISO methodologies (UNE-EN:ISO6579:2002) for the detection of *Salmonella* spp. and furthermore serotyped by means of agglutination techniques using commercially available antisera (Statens Serum Institut, Denmark) and a phage's panel provided by the International Reference Laboratory of phage typing (Colindale, London, England).

III. RESULTS

A significant reduction in *Salmonella* spp. prevalence was observed between 2010 and 2013 at all stages of production, interestingly *Salmonella* were not recovered from meat samples in 2013. Comparatively, a total of 39 *Salmonella* spp. isolates were recovered from 375 samples (10.40%) in 2013, while 76 *Salmonella* spp. were isolated from 400 samples (19%) in 2010 at same stages of the production chain (Hernández *et al.*, 2013). However, an increase in prevalence was noted in tonsil samples (17.50% *vs.* 29.34%) over the same time period. As the presence of *Salmonella* in the tonsils can be present from as little as 30 minutes after internalisation of the pathogen (Vieira-Pinto *et al.*, 2006), the source of the contamination could have occurred before or during lairage.

In 2010, 14 different serotypes were isolated: Bredeney (37 strains), Rissen (35), Derby (25), Typhimurium (11), Montevideo (4), Israel, (4) Anatum (2), Emek, (2) Monophasic *Salmonella* Typhimurium (mST) (1), Choleraesuis (1), Durban (1), Kentucky (1), London (1) and Sandiego (1). *S.* Typhimurium phage types U311 (8), 193 (1), 104b (1) and UT (1) and mST phage types U311 were detected. In 2013, 13 different serotypes were isolated: mST (21), Anatum (20), Typhimurium (17), Hessarek (15), Derby (8), Newport (7), Kentucky (3), Bredeney (1), Infantis (1), Rissen (1), Veneziana (1), Rubislaw (1) and Heidelberg (1). *S.* Typhimurium phage types 104B (2), 104L (2), 193 (11), U302 (1) and U311 (1) and mST phage types 104B (1), 104L (2), 120 (1), 138 (2), 193 (6), U302 (7), U311 (1) and NT (cannot be defined) were identified.

	Year of study	
Sample	**2010**	**2013**
Skin (abrasive sponge)	29/80 (36.25)	2/75 (2.67)
Ileocaecal lymph nodes	13/80 (16.25)	4/75 (5.34)
Faeces	17/80 (21.25)	11/75 (14.67)
Tonsils	14/80 (17.50)	22/75 (29.34%)
Meat	3/80 (3.75)	0/75(-)
TOTAL	19.00%	10.40%

Table 1. Number (positive/tested) and prevalence (%) of *Salmonella* spp. in a free a free-range pig slaughterhouse

IV. CONCLUSIONS

- **Our results show the isolation of different serotypes of *Salmonella* spp. from different stages of the slaughterline. Furthermore, a shift in *Salmonella* serotypes was detected to a higher prevalence of mST, *S.* Typhimurium and *S.* Hessarek in 2013.**
- **The intensification of disinfection protocols applied after 2010 results (i.e. hydroalcoholic disinfection spraying protocols for cutting surfaces at different intervals) may represent a useful control measure to minimize *Salmonella* circulation in pork. However, more studies are necessary to identify other critical points in reducing cross-contaminations at the slaughterhouse.**

Diversity of *Salmonella* spp. and *Listeria monocytogenes* isolates from free-range pig production plants. Morales A., Cardoso-Toset F., Luque I., Tarradas C., Astorga R.J., Herrera León S., Hernández M., Gómez-Laguna J., *9th European Symposium of Porcine Health Management ESPHM*. Edimburgo, 2013, Prague , Czech Republic, 2017

3th - 5th May 2017 Prague, Czech Republic

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Diversity of *Salmonella* spp. and *Listeria monocytogenes* isolates from free-range pig production plants

Morales A.[1], Cardoso-Toset F.[1,2], Luque I.[2], Tarradas C.[2], Astorga, R.J.[2], Herrera-León, S.[3], Hernández M.[1], Gómez-Laguna J.[1,4]

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I. INTRODUCTION AND OBJECTIVE

Zoonotic agents, such as *Salmonella* spp. and *Listeria monocytogenes*, are considered high-risk zoonotic pathogens by the European Food Safety Agency (EFSA) and have a significant public health impact. In this sense, it is of high interest to gain knowledge in the evolution of the different serotypes and clones to develop updated control measures. Thus, the aim of this study was to evaluate **the diversity of *Salmonella* spp. and *Listeria monocytogenes*** strains isolated along the pork chain in two different slaughterhouses.

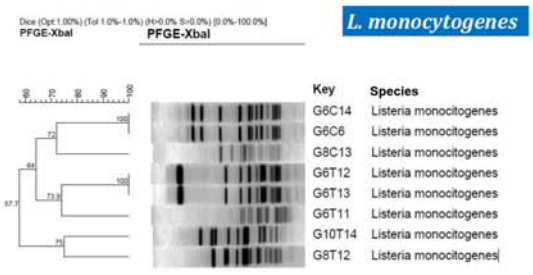
II. MATERIAL & METHODS

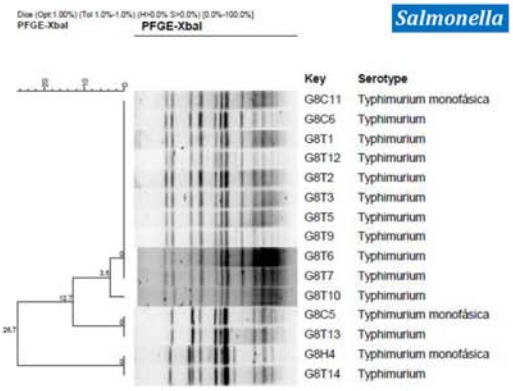
A total of 150 animals (fifteen animals/farm) were traced at both slaughterhouses to collect a total of five samples/animal (faeces, abrasive sponges at the pre-scalding point, ileocolic lymph nodes, tonsils and a pool of meat samples), that were analysed using specific ISO methodologies for the detection of *Salmonella* spp. and *Listeria monocytogenes*. Bacteria serotype was determined by means of an agglutination technique using commercially available antisera and *Salmonella* phage type by means a phage´s panel provided by the International Reference Laboratory of phage typing. The genetic similitude of the isolates was evaluated by PFGE analysis.

III. RESULTS

The global prevalence of *Salmonella* (97 isolates) and *L. monocytogenes* (68 isolates) was of 12.93% and 9.07%, respectively. Thirteen different *Salmonella* serotypes were detected with monophasic *Salmonella* Typhimurium (mST) (21), Anatum (20), Typhimurium (17) and Hessarek (15), as the most frequent ones. The phage types 193 (17) and U302 (8) were the most common ones. Thirty-nine *L. monocytogenes* isolates were identified as serotype 4b and 28 samples identified as serotype 1/2a, with the three isolates recovered from meat samples belonging to the latter.

According to the results of PFGE analysis, four different pulsotypes of *Salmonella* were identified, with the same clone being detected from meat and tonsil samples, which point out potential cross-contamination. Six different pulsotypes of *L. monocytogenes* were detected, but no evidence of cross-contamination was detected.





DISCUSION & CONCLUSIONS

Our results highlight a high diversity in *Salmonella* and *L. monocytogenes* isolates recovered from free-range pig samples, with evidence to cross-contamination along the production chain in the case of *Salmonella*.

ACKNOWLEDGEMENTS

This study was financially supported by the Department of Innovation, Science and Enterprise of the regional government of Andalusia (Agencia IDEA), Spain (project number #351504)

Survival of *Salmonella*, *Campylobacter* and *Listeria* on dry cured pork loins. Morales-Partera, A.; F. Cardoso-Toset, F. Jurado-Martos, R.J. Astorga, B. Huertas, I. Luque, C. Tarradas, J. Gómez-Laguna. *Interanual Symposium Salmonella and Salmonellosis*, Saint Malo, France 2018.

SURVIVAL OF *Salmonella*, *Campylobacter* AND *Listeria* ON DRY CURED PORK LOINS

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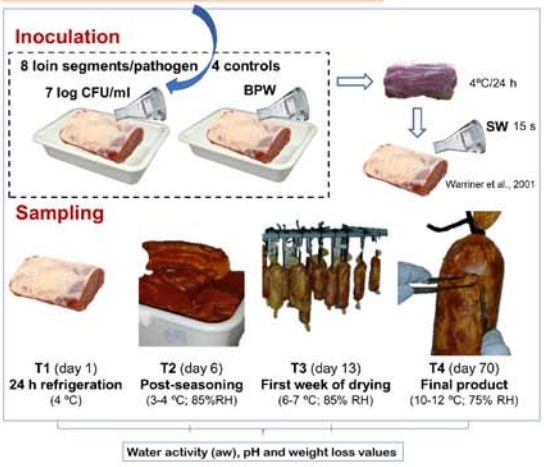
I. INTRODUCTION

The safety of ready-to-eat products such as cured pork loins must be guaranteed by the food industry. Foodborne pathogens such as *Salmonella* spp., *L. monocytogenes* and *Campylobacter* spp. have been shown to survive some fermentation, maturation and drying procedures necessary to obtain dried and fermented pork products being ocasionally detected in markets and specialty food shops. In the present study, the efficacy of the dry curing process of pork loins obtained from free-range pigs in the reduction of three of the most important foodborne pathogens is analysed.

II. MATERIAL & METHODS

- Monophasic *Salmonella* Typhimurium 1,4,[5],12:i:- DT 193 (mST)
- *Campylobacter coli*
- *Listeria innocua* serotype 6a CECT 910 (STCC, Spain)



III. RESULTS

Physicochemical parameters (pH, a_w and weight loss) evaluated in dry-cured loins

A progressive reduction of pH and a_w was obtained from T1 to T4, with a final average pH value of 5.56 ± 0.12 and a final average a_w value of 0.887 ± 0.03 ($P < 0.05$). As expected, the average weight loss value reached around 40% when dry-cured loins were fully processed (Table 1).

Table 1. Changes on physicochemical parameters (pH, a_w and weight loss) along the dry-curing process of loins. Results are expressed as means ± standard deviation.

Parameter	Mean ± SD
pH	
T1	5.85 ± 0.18[a]
T2	5.84 ± 0.20[a]
T3	5.79 ± 0.14[a]
T4	5.56 ± 0.12[b]
a_w	
T1	0.986 ± 0.00[a]
T2	0.980 ± 0.00[b]
T3	0.960 ± 0.01[c]
T4	0.887 ± 0.03[d]
Weight loss (%)	
T4	40.81 ± 3.02

T1: 24-hour post inoculation; T2: post-additive; T3: end of the first week of drying; T4: final product. [a-d] Values within a column with different superscripts differs significantly at $P < 0.05$.

Selected foodborne pathogens during the dry-cured processing of pork loins

The results show a significant drop of *S.* Typhimurium ($P = 0.03$) over the curing period with a percentage reduction of 62.68 % (3.28 log CFU/g) from the initial phase till the final product (Fig. 2). The counts for *L. innocua* fluctuated between the different stages of analysis with an overall no significant reduction of 7.69 % (0.48 log CFU/g) with respect to the initial stage (Fig. 2).

C. coli counts before the loins were macerated were of 2.14 ± 0.19 log CFU/g. The final product was assessed to be absent for *C. coli* indicating a 100 % reduction over the production process ($P = 0.03$) (Fig. 2).

No pathogens were detected in control samples at the different analyzed stages by using the enumeration and detection methods.

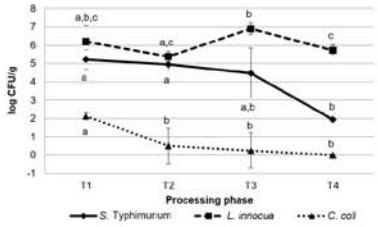


Fig. 2. *S.* Typhimurium, *L. innocua* and *C. coli* average bacterial load reduction along dry cured loins processing. T1: 24-h post-inoculation. T2: post-additive. T3: end of the first week of drying. T4: final product.

IV. DISCUSSION

According to the literature, the dry curing process generally inhibits the proliferation of foodborne pathogens due to the concurrence of several microbial hurdles, such as a low pH and aw, a high salt concentration, the addition of nitrites, spices and other ingredients or the growth of competitive flora that contribute to the final product stability (Stollewerk *et al.* 2012).

The results of the present study show that these bacteriostatic parameters are able to reduce the load of some foodborne pathogens, such as *S.* Typhimurium and *C. coli*, after processing of dry cured Iberian pork loins. However, considering that the complete elimination of *S.* Typhimurium and *L. innocua* was not obtained, the initial concentration of these pathogens on fresh loins may be considered as a critical point to obtain safe ready-to-eat dry cured loins.

Although the results obtained in this study characterise the effectiveness of the dry curing process in reducing some pathogenic bacteria, such as *S.* Typhimurium and *C. coli*, future studies which include different strains of each pathogen would be valuable to rule out the possibility of intra-species variability in the susceptibility of the inoculated pathogens to the dry curing process.

V. CONCLUSIONS

Logarithmic levels of *S.* Typhimurium and *C. coli* declined over the production process of dry cured pork loins, whereas only mild changes were observed for *L. innocua* counts. Three critical factors to justify these findings were observed: (i) the initial concentration of the bacteria; (ii) the progressive reduction of pH; and (iii) the reduction of a_w values. Although a reduction of bacterial counts throughout the process is observed, the results of this study do not support that the production process of dry-cured pork loins completely removes *S.* Typhimurium and *Listeria monocytogenes* in case of high initial contamination.

❑ The present study has been published in *International Journal of Food Microbiology* (2017) 258, 68-72.

Agradecimientos

Son muchas las cosas que se me han pasado por la cabeza a lo largo de este proyecto de tesis doctoral pero sin lugar a dudas me quedo con la satisfacción de compartir esta experiencia con muchas personas que se han volcado y me han transmitido su saber hacer.

Debo agradecer de manera especial su compromiso, dedicación y esfuerzo a mis directores de tesis doctoral, los doctores Carmen Tarradas Iglesias, Jaime Gómez Laguna y Fernando Cardoso Toset.

Carmen, gracias por haber decidido compartir tus conocimientos. Sabes lo complicado que ha sido compaginar, sobre todo estos últimos años, la tesis con el emprendimiento. Gracias por motivarme cada día para continuar sin tirar la toalla. Sin ti esto no habría sido posible, te agradezco enormemente la confianza que depositaste en mi por tu frase de "Esto Ángela, como tú sabes hacerlo". **Jaime,** para mi eres un referente en lo personal y profesional. Confiaste en mí desde el primer día que comenzamos esta andadura. Gracias por tu apoyo incondicional y por hacerme crecer día a día como profesional. Contigo es siempre fácil trabajar por lo que espero que esto solo sea un comienzo de nuestro camino juntos. **Fernando,** gracias por tu ayuda y apoyo en mis momentos de crisis. En esta tesis, nadie mejor que tú para cargarse con decisión de un plumazo los interminables textos de prosa de una servidora. Te admiro como profesional, uno de mis científicos favoritos.

Dar las gracias al **Departamento de Sanidad Animal de la Universidad de Córdoba,** Inmaculada Luque, Rafael Astorga, Alfonso Maldonado, Belén Huerta, Ángela Galán, Belén Barrero, Fabiana Aguilar, Francisco Jurado y Ana Lucía Solarte.

Inma, un placer trabajar contigo, siempre disponible ante cualquier contratiempo. Gracias por tu contribución en este trabajo.

Agradecer al **Centro de Investigación y Calidad Agroalimentaria del Valle de los Pedroches (CICAP)** el poner a disposición de este proyecto los medios necesarios para llevarlo a cabo. **Layla Fernández**, continuaras siendo mi jefa. Siempre exigente, con el objetivo de sacar lo mejor de mí. Hoy día puedo decir que gracias a las lágrimas, noches sin dormir y a tu confianza me enfrenté a retos que me hicieron crecer como persona y profesional. Muchas gracias por una de las experiencias más enriquecedoras de mi vida.

Dar las gracias **The Built Environment and Sustainable Technologies (BEST) Research Institute, Faculty of Technology and Environment, Liverpool John Moores University** en especial a Montse, Eduardo y Sam por su acogida durante mi estancia en el centro y a Ahmed, Alex y Richard por transmitirme sus conocimientos.

Dar las gracias a los **técnicos y operarios** que facilitaron el proceso de selección y recolección de muestras.

A **todos mis amigos** por soportar y apoyar la historia interminable de Tesis doctoral.

Agradecer a mi **tía Lola** y **Maruja** su apoyo constante y ver siempre el lado positivo de las cosas.

Por último, agradecer a **mis padres y a mi hermano** apoyarme en cada decisión y proyecto y permitirme cumplir el desarrollo de esta tesis. A mi padre, gracias por haberme transmitido el valor de implicarse y sacrificarse por lo que uno quiere, creo que “ahora si te he superado”. A **mi madre** agradecerle su constante paciencia.

A todos, muchas gracias

Listado de abreviaturas / List of abbreviations

ADG	Average Daily Gain
aW	Water activity
BAL	Bacterias ácido lácticas
BPW	Sterile Buffered Peptone Water
CFU	*Colony-forming unit*
CI_{95}	*95% confidence intervals*
EFSA	*European Food Safety Authority*
ELISA	*Enzyme linked immunosorbent assay*
ETAs	*Enfermedades Transmitidas por Alimentos*
GMP	*Good Manufacturing Practices*
HACCP	*Hazard Analysis Critical Control Point*
MAPAMA	Ministerio de Agricultura, Pesca, Alimentación y Medio Ambiente
NaCl	*Sodium chloride*
NC	*Negative cases*
NIR	Near Infrared Spectroscopy
OD_{600}	*Optical density measured at a wavelength of 600 nm*
PFGE	*Pulsed Field Gel Electrophoresis*
PCR	*Polymerase chain reaction*
qPCR	*Real-time polymerase chain reaction*
RH	*Relative humidity*
RTE	*Ready-to-eat*
Se	*Sensitivity*
SD	*Standard desviation*
SSOP	*Sanitation Standard Operating Procedures*
SW	Sterile Water
UE	Unión Europea